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The development of vaccine approaches for breast cancer has the potential to provide an adjuvant therapy with low toxicity for patients at risk for disease recurrence. Polynucleotide vaccines have several advantages compared to traditional vaccines including the ability to elicit antigen-specific T cells, inherent immunogenicity, ability to modify the encoded antigen, and excellent safety profile. However, clinical efficacy has been disappointing and strategies to enhance the potency of polynucleotide vaccines are needed. Adenoviral vectors also show promise for cancer vaccine approaches, but do not efficiently deliver genes to antigen presenting cells such as dendritic cells (DCs). We are investigating novel vaccine strategies for breast cancer. The target tumor antigen is carcinoembryonic antigen (CEA), which is highly expressed in most breast tumors. DCs show promise for cancer immunotherapy due to their critical role in mediating immune response. Development of an optimal DC transduction protocol for tumor antigen presentation would represent a significant advancement in DC-based vaccination strategies. We are investigating methods of DC transduction and antigen modification that will elicit the most potent anti-tumor immune response. These studies have employed a nontransgenic mouse model of adenocarcinoma as well as a more stringent transgenic model.

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Novel Vectors for Dendritic Cell Transduction
DAMD17-00-1-0122
Principal Investigator: Theresa V. Strong, Ph.D.
Final Report June 1, 2000 – May 31, 2003

INTRODUCTION:

Cancer immunotherapy approaches aim to enhance the cellular immune response against tumor antigens. Although cytolytic T cells specific for tumor antigens can be isolated from tumor-bearing individuals, it is clear that immune system fails to produce effective antitumor immunity. In recent years, dendritic cells (DCs) have received much attention as their critical role in the elicitation of immune response has been appreciated. Preclinical studies and initial clinical trials using these cells for tumor antigen presentation have produced some encouraging results, however, gene transfer technology for DCs has not yet been optimized. Polynucleotide vaccines offer an alternative to traditional protein vaccines or viral-based vaccines. Advantages of polynucleotide vaccines include the ability of these vaccines to elicit tumor antigen-specific cytolytic T cells, the inherent immunogenicity of plasmid DNA or replicative RNA vaccines, and the favorable safety profile as demonstrated by human clinical studies. The limitation of polynucleotide vaccines has been their lack of potency, as demonstrated by their limited ability to break immunological tolerance. The goal of this project was to develop and evaluate novel vector systems for cancer immunotherapy. Naked RNA has been shown to mediate gene transfer into dendritic cells and we hypothesize that the use of replicative RNA will enhance transgene expression and improve tumor antigen presentation. We are also studying a targeted adenoviral vector developed by our colleagues in the Gene Therapy Center at the University of Alabama at Birmingham (1). These adenoviral vectors are specifically targeted to the CD40 molecule present on dendritic cells. The target tumor antigen we are studying is CEA, which is highly expressed on human breast cancer and has several features that make it an attractive target for immunotherapy (2). These include its high level expression in most breast tumors, as well as other epithelial tumors, and its probable role in tumorigensis. As a model system to evaluate these vectors, we are using two mouse models; a syngeneic tumor cell line expressing CEA implanted into a wild type mouse, and a more stringent CEA-transgenic mouse model which allows us to evaluate the ability of these vaccines to break immunological tolerance, induce a CEA-specific immune response and mediate an effective antitumor immune response. The approved specific aims of this project are:

1. To evaluate the ability of replicative RNA vectors encoding CEA to transfect dendritic cells ex vivo, and elicit an antitumor immune response in a CEA transgenic mouse model of adenocarcinoma.

2. To use a bispecific antibody to produce a CD40-targeted adenovirus encoding CEA, and to evaluate its specificity in transducing dendritic cells and efficacy in inducing an antitumor immune response in a CEA transgenic mouse model.

BODY:

Transgenic CEA animals: We initially experienced some difficulties in establishing a successful breeding colony of CEA transgenic mouse line at our institution (reported year 2), but have overcome that problem and are now reliably breeding these animals.

Evaluation of dendritic cell populations following delivery of plasmid DNAs encoding cytokines and chemokines. Although our proposal calls for ex vivo delivery of RNA and adenoviral vectors to dendritic cells, it would be highly advantageous to deliver to RNA in vivo, if possible. Ex vivo cultures are expensive, difficult to maintain, and labor intensive. Such an approach would therefore be difficult to translate readily to the clinical setting. To determine if plasmid DNA can be used to effectively recruit DC and other immune cells for in situ delivery of our vaccines, we have used a combination of immunohistochemical staining and quantative real-time PCR (QRT-PCR) to assess the immune cell infiltration and cytokine production following intramuscular injection of plasmids, including pGM-CSF and pSDF1-α. These studies have shown a different pattern of infiltration, with GM-CSF attracting primarily type 1 dendritic cells and macrophages, while SDF1-α attracts primarily type 2 dendritic cells and macrophages (Table 1 and Fig. 1). Cytokine profiles as measured by QRT-PCR also differ between muscles injected with pGM-CSF versus pSDF-1□ (Fig. 2). These studies are currently ongoing, evaluating the time course of cytokine message for IL-2, IL-4, IL-10, IL-12, GM-CSF and IFNγ in muscles injected with different doses and formulations of cytokine/chemokine plasmids.

Table 1. Summary of cellular infiltrate experiments

Day	Empty vector	SDF-1α	GM-CSF
1	+ Macrophages + PMNs	+ Macrophages + PMNs	+ Macrophages + PMNs
3	+ Macrophages + PMNs	++ Macrophages + DC2	++ Macrophages ++ DC1
7	+++ Macrophages ++ PMNs	++ Macrophages ++ DC2	+++ Macrophages ++ DC1
14	+ Macrophages + PMNs	+ Macrophages + DC2	++ Macrophages ++ DC1
21	+ Macrophages	+ Macrophages	+ Macrophages + DC1

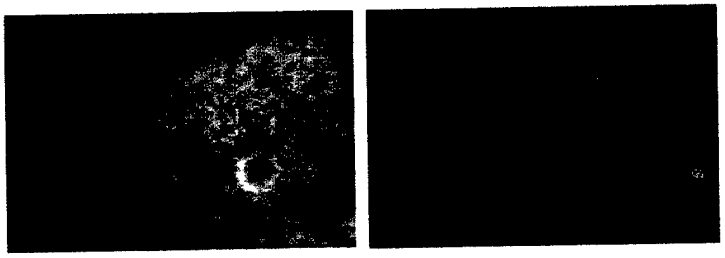

Figure 1. Immunohistochemical analysis of pGM-CSF (left) and pSDF-1a (right) muscle 7 days after i.m. injection. Sections were stained with anti-CD11c (green), anit-CD4 plus CD8 (red), and anti-IgM (blue).

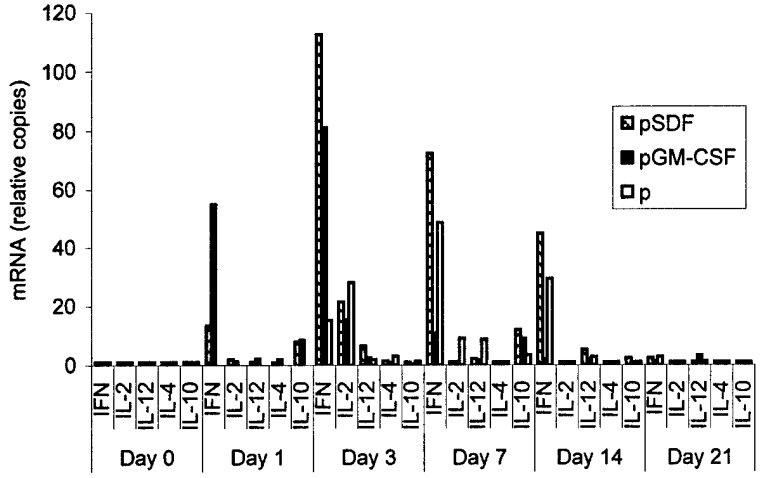

Figure 2. Time-course of cytokine mRNA production for mice injected i.m. with plasmids expressing SDF-1⇨ or GM-CSF. QRT-PCR for IFN→, IL-2, IL-12, IL-4 and IL-10 was performed with RNA extracted from muscles injected with 50 ↘g plasmid encoding SDF-1⇨ (pSDF), GM-CSF (pGM-CSF) or empty vector (p). Day 0 represents cytokine mRNA of uninjected muscle.

Evaluation of genetic fusions of CEA and GM-CSF. Prior to it's incorporation into a replicative RNA vector, we evaluated methods to enhance the immunogenicity of the encoded CEA molecule. These studies included the incorporation of know CD4+ T cell helper epitopes into the transgene (reported year 2) and incorporation of the cytokine GM-CSF as a fusion molecule with CEA (**see Lima et al, appended**). These studies have demonstrated that a fusion of CEA with GM-CSF is more antigenic that CEA alone, but that the potential exists for induction of an anti-GM-CSF immune response that might undermine the vaccine efficacy. Thus, careful control of vaccine dose and schedule will be needed to achieve the optimal efficacy.

Replicative RNA vectors for Breast Cancer Immunotherapy. We have produced replicative RNA vectors encoding CEA with and without GM-CSF. The fusion constructs, described above and in Lima et al, appended, have been cloned into the Semiliki forest virus vector. We plan to proceed with the evaluation of these vectors in the transgenic mouse model, as described in the original proposal.

Novel Adenoviral Vectors for Breast Cancer Immunotherapy. Our collaborators (DT Curiel and co-workers) in the Gene Therapy Center have produced a CD40 targeting molecules to enhance the ability of adenoviruses to transduce CD40 expressing cells, such as dendritic cells (1, 3, and 4-appended). Although prior work with a bispecific antibody (as specified in the proposed **specific aim 2**) showed some promise in previous studies, newly developed targeting molecules have significant advantages compared to the bispecific antibody proposed in the original application. These advantages include comparative ease of preparation, the uniformity of preparation and the resulting targeted Ad preparation, and the stability of the adenoviral-targeting molecule complex. For these reasons, we chose to abandon the bispecific antibody, which proved difficult to work with, in favor of newly developed targeting molecules now available. One soluble molecule consisting of coxsackie adenoviral receptor (CAR) ectodomain fused to a single chain antibody recognizing CD40 (CAR/G28) was evaluated for its ability to CD40 expressing cells using reporter genes (3, 4-appended). Another novel soluble CD40-targeting fusion molecule has also been developed and is currently being evaluated by our collaborators using an adenovirus encoding a reporter gene. Relevant to our studies, an adenovirus encoding human CEA has also been produced (5). Thus, we will use these newly validated adenoviral targeting molecules with the AdCEA in the CEA-transgenic mouse model.

KEY RESEARCH ACCOMPLISHMENTS

1. Developed a mouse DC preparation protocol for reliable production of mouse DCs (reported year 1).
2. Produced a truncated CEA antigen, evaluated in a nontransgenic mouse model of adenocarcinoma, demonstrating equivalency with full length CEA (Reported year 2).
3. Evaluated incorporation of T-helper epitopes into CEA (Reported year 2).
4. Established CEA-transgenic breeding colony (Reported year 2).
5. Evaluated the ability of plasmid DNA delivery of chemokines and cytokines to recruit DC and other immune cells for in situ delivery of vaccines.
6. Evaluated a CEA-fusion construct including mGM-CSF and determined that the encoded fusion protein can elicit a strong immune response, but may also induce anti-GM-CSF immune response (manuscript appended).
7. In collaboration with the Gene Therapy Center at UAB, obtained novel soluble CD40-targeting molecules for enhanced efficiency of adenovirus mediated DC transduction. These molecules will now be applied for immunotherapy in the CEA transgenic model.

REPORTABLE OUTCOMES

Several novel DNA, RNA, and adenoviral constructs have been produced as a result of this project. Details of these will be included in publications, and these reagents will be freely distributed to any interested investigator in accordance with the policies of the USAMRMC, the NIH and the University of Alabama at Birmingham Technology Transfer Office.

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Abstracts:

Lima, J., Jenkins C, Hamilton M, Triozzi P, Shaw DR, Strong TV. A DNA vaccine encoding genetic fusions of CEA and GM-CSF. Abstract presented orally at the Society for Biological Therapy (2001):

Lima J, Jenkins C, Triozzi PL, Shaw DR, Strong TV. Development of a more potent DNA vaccine for breast cancer. Era of Hope Meeting, 2002.

Lima J, Allen K, Aldrich W, Shaw DR, Strong TV, Triozzi PL. Differential effects on cytokine production and dendritic cell (DC) subpopulations in situ of plasmids encoding GM-CSF, SDF1a, and carcinoembryonic antigen (CEA). Proc Am Assoc Cancer Res 44:947, 2003 (Abstract#4766)

Manuscripts:

Lima J, Jenkins C, Guerrero A, Triozzi PL, Shaw DR, Strong, TV. A DNA vaccine encoding genetic fusions of carcinoembryonic antigen (CEA) and granulocyte macrophage colony stimulating hormone (GM-CSF). J Immunother (submitted, in revision)

Strong TV. Polynucleotide Immunization for Cancer Therapy in *Cancer Gene Therapy* DT Curiel and JT Douglas, eds., Humana Press, Totowa, NJ (in press)

CONCLUSIONS

Although we have not completely accomplished the original specific aims proposed, our studies have led to the development of several novel vectors for breast cancer immunotherapy. Work on the specific aims outlined in the proposal will continue beyond the scope of this grant and will be focused

on the evaluation of the vectors in the CEA transgenic model. The ultimate goal of the studies is to render more clinically efficacious the promise of vaccine therapy for the treatment of breast cancer. Polynucleotide and adenoviral vaccines for cancer immunotherapy have many aspects favoring their development as vectors for inducing antitumor immune responses. We anticipate that these vaccines would be clinically applicable to breast cancer patients at high risk for disease recurrence. As an adjuvant therapy, this strategy potentially offers a potentially low toxicity approach for the elimination of micrometastatic disease. We anticipate that the continued development of these vaccines improve the clinical effectiveness of this therapeutic modality.

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PERSONNEL WHO RECEIVED SALARIES FROM THIS GRANT:

Name	Role
Theresa Strong	PI
Denise Shaw	Co-Investigator
Jose Lima	Postdoctoral fellow
Connie Jenkins	Research assistant
Zhana Muminova	Research associate

Appendices:

All bibliography items are included in the Appendix:

Abstracts:
Lima, J., Jenkins C, Hamilton M, Triozzi P, Shaw DR, Strong TV. A DNA vaccine encoding genetic fusions of CEA and GM-CSF. Abstract presented orally at the Society for Biological Therapy (2001):

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Also included: Hakkarainen et al, Clin Cancer Res 9:619-624, 2003.

A DNA Vaccine Encoding Genetic Fusions of CEA and GMCSF.

Jose Lima, Connie Jenkins, Mary Hamilton, Pierre Triozzi, Denise Shaw, and Theresa Strong.
Department of Medicine and the Comprehensive Cancer Center, The University of Alabama at Birmingham, Birmingham, AL.

Plasmid DNA vaccines encoding tumor antigens have shown promise in animal models, but limited efficacy in the clinical setting. We are investigating the use of plasmid DNA encoding fusion proteins to better target antigen presenting cells for enhanced immune response. As a first step, we constructed two plasmids encoding fusions between carcinoembryonic antigen (CEA) and murine GM-CSF (mGM-CSF). These constructs used a shortened, secretory form of CEA that has an in-frame deletion removing the second of the three highly homologous repetitive segments of CEA. This truncated CEA was fused with GM-CSF in the carboxy or amino terminal, with a short, flexible linker joining the two moieties. *In vitro* studies validated that the fusion proteins were produced, secreted and recognized by both anti-CEA and anti-GM-CSF antibodies. GM-CSF activity was confirmed with a GM-CSF dependent mouse cell line. Immunization of C57/BL6 mice with DNAs encoding the fusion proteins led to T cell and antibody responses against CEA. These responses were comparable to immunization with plasmid DNA encoding full length CEA only. Tumor challenge with CEA-expressing syngeneic mouse adenocarcinoma cells (MC38-CEA) led to the development of large tumors in control groups by day 25. In contrast, no tumors were noted in any of the CEA or CEA-GM-CSF immunized groups at this time. Subsequently, tumors developed at approximately day 35 in those animals immunized with the CEA-GM-CSF fusions (5/10 and 7/10 of animals in each group), while only 1/20 in the plasmid CEA alone groups developed tumors. Further evaluations demonstrated that mice injected with the CEA-GM-CSF fusion plasmids developed IgG autoantibodies to mGM-CSF, and that these antibodies neutralized mGM-CSF activity *in vitro*. Mice injected with plasmid DNA encoding GM-CSF alone did not produce such antibodies. A single, low dose immunization with fusion plasmids resulted in lower titers of anti-mGM-CSF antibodies better tumor protection than CEA encoding plasmid alone.

DEVELOPMENT OF A MORE POTENT DNA VACCINE FOR BREAST CANCER

Jose Lima, Connie Jenkins, Pierre L. Triozzi, Denise R. Shaw, and Theresa V. Strong

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DNA vaccines are being developed for a number of clinical applications, including cancer immunotherapy. Immunization with DNA encoding tumor antigens elicit effective antitumor immune responses in mouse models, but thus far show limited efficacy in the clinical setting. We are exploring strategies to enhance the efficacy of nucleic acid vaccines for breast cancer immunotherapy, using carcinoembryonic antigen (CEA) as a model antigen. To better target the encoded antigen to the appropriate antigen presenting cells, we constructed plasmids encoding fusions between CEA and murine GM-CSF (mGM-CSF). In vitro studies validated that the fusion proteins were produced, secreted and recognized by both anti-CEA and anti-GM-CSF antibodies. GM-CSF activity was confirmed with a GM-CSF dependent mouse cell line. Immunization of C57/BL6 mice with DNAs encoding the fusion proteins in a high dose immunization schedule led to T cell and antibody responses against CEA, which were comparable to immunization with plasmid DNA encoding full length CEA. Tumor challenge with CEA-expressing syngeneic mouse adenocarcinoma cells (MC38-CEA) led to the development of large tumors in control groups, protection from tumor growth in mice immunized with plasmid encoding CEA only, and growth of tumors at a late timepoint in mice immunized with the CEA-GMCSF fusion constructs. Further evaluation demonstrated that mice injected with the CEA-GMCSF fusion plasmids developed IgG autoantibodies to mGM-CSF, and that these antibodies neutralized mGM-CSF activity in vitro. However, a single, low dose immunization with fusion plasmids resulted in lower titers of anti-mGM-CSF antibodies and provided better tumor protection than CEA encoding plasmid alone. Ongoing studies will evaluate the ability this modified CEA vaccine to elicit an antitumor immune response capable of breaking immunological tolerance and providing tumor protection in a CEA-transgenic mouse model. The ultimate goal of these studies is to develop an effective cancer vaccine to be adminstered at the time of primary intervention, thereby preventing recurrence and metstatic disease.

The U.S. Army Medical Research Materiel Command under DAMD17-00-1-0122 supported this work.

#4766 Differential effects on cytokine production and dendritic cell (DC) subpopulations in situ of plasmids encoding GM-CSF, SDF-1α, and carcinoembryonic antigen (CEA). Jose Lima, Karen Allen, Wayne Aldrich, Denise R. Shaw, Theresa V. Strong, and Pierre L. Triozzi. *The University of Alabama at Birmingham, Birmingham, AL.*

DNA vaccine strategies that incorporate tumor antigens plus cytokines and chemokines have demonstrated variable immunoprotective antitumor effects. To develop more efficacious DNA cancer vaccines, plasmids encoding the tumor antigen CEA, the chemokine SDF-1α, the cytokine GM-CSF, and a CEA-GM-CSF fusion protein were studied for the ability to modulate cytokine production and to recruit DC populations in situ. Mice were injected i.m. with 50 ug of these plasmids as well as an empty vector plasmid. Production of IFN-γ, IL-2, IL-4, IL-10, and IL-12(p40) was determined by quantitative real time polymerase chain reaction of mRNA extracted from the injected muscle. Cellular infiltrates were characterized by hematoxylin and eosin (H&E) staining and by immunofluorescence microscopy for markers of DC1, DC2, macrophage, T cells and B cell populations. The injection of all plasmids, including empty vector, elicited IFN-γ, IL-2, and IL-12(p40) production at days 3 to 5. Production of these cytokines peaked at days 5 to 10 and was greater with pCEA and pSDF-1α than with pGM-CSF or with the pCEA-GM-CSF fusion. IL-10 production peaked at days 10 to 14. Significant production of IL-4 was not observed. IFN-γ production with pCEA was higher and more prolonged than that with the pCEA-GM-CSF fusion. Tumor protection experiments using the MC38-CEA-2 tumor model (C57BL/6 mice) demonstrated superior protection with pCEA alone compared to the pCEA-GM-CSF fusion following three immunizations with 50 ug of plasmid. The cellular infiltrate, consisting of macrophages, polymorphonuclear neutrophils, lymphocytes, and DC, as seen on H&E staining, progressively increased in all injected mice and peaked on day 7. After that time, cellular infiltrates decreased in all samples, although at a faster rate in the empty vector and pSDF-1α injected mice. By day 21 only the pGM-CSF injected muscle had an inflammatory infiltrate of significant size. Macrophages (CD11b) and DC1 (CD11bCD11c) were elicited by pGM-CSF. In contrast, DC2 (CD11c+), T cells (CD4/CD8+) and B cells (IgM+) were elicited by pSDF-1α. Significantly more macrophages were elicited with pGM-CSF and with the pCEA-GM-CSF fusion. These studies show differential effects of the SDF-1α, GM-CSF, CEA, and CEA-GM-CSF plasmids on cytokine production and DC subpopulations in situ and also provide data regarding the kinetics of these effects that can be applied to optimize DNA cancer vaccine strategies.

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* Title Page

A DNA Vaccine Encoding Genetic Fusions of Carcinoembryonic Antigen (CEA) and Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF)

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ABSTRACT

Plasmid DNA vaccines are being developed for a number of clinical applications, including cancer immunotherapy. Here, the anti-tumor immunologic effects of DNA vaccines expressing fusion proteins of granulocyte/macrophage colony-stimulating factor (GM-CSF) and the human tumor antigen, carcinoembryonic antigen (CEA), were examined. Plasmids with mouse GM-CSF fused at the amino or carboxy terminus of a truncated CEA were constructed. Studies *in vitro* demonstrated that fusion constructs of both orientations produced the expected proteins with GM-CSF biologic activity. Immunization of C57BL/6 mice with the CEA-GM-CSF fusion plasmids in a three injection, high-dose immunization schedule led to T cell and antibody responses specific for CEA. Mice injected with CEA-GMCSF fusion plasmids also developed IgG autoantibodies to GM-CSF, whereas mice administered plasmid encoding GM-CSF alone did not. Tumor challenge with the CEA-expressing syngeneic mouse adenocarcinoma line, MC38-CEA-2, showed delayed tumor growth in mice immunized with the CEA-GM-CSF fusion plasmids but complete protection in mice immunized with plasmid encoding CEA alone. In contrast, a single low dose immunization with CEA-GM-CSF fusion plasmids provided better tumor protection than low dose CEA plasmid alone and resulted in lower titers of GM-CSF antibodies. Thus, immunization with CEA-GM-CSF fusion plasmids can induce effective anti-tumor immune responses, but can also induce autoantibodies to GM-CSF. GM-CSF autoantibody induction may be modified by immunization dose and schedule. GM-CSF autoantibody elicited with low dose CEA-GM-CSF fusion plasmid immunization did not abrogate the ability of immunized mice to reject tumor challenge.

Key words: plasmid vaccine, immunotherapy, CEA, GM-CSF.

INTRODUCTION

Plasmid DNA vaccines encoding tumor-associated antigens have emerged as a potentially nontoxic adjuvant therapy for cancer. DNA immunization offers several advantages compared to other types of vaccines. Chief among these is intracellular production of the immunogen, leading to the induction of long-term cell mediated immunity (1). DNA vaccines are also relatively easy to prepare, stable, and comparatively inexpensive. Importantly, they do not induce vector immunity, making repeat dosing feasible, and they have inherent adjuvant effects due to the presence of unmethylated CpG dinucleotides (2). Phase I trials have shown that DNA vaccines are generally safe and well tolerated and evidence of immune response has been demonstrated for several vaccines (3-6). Recently, we reported the results of a phase I study using plasmid DNA-encoded carcinoembryonic antigen (CEA), a human tumor antigen which is overexpressed by many common cancers (7). In this trial in patients with advanced colorectal carcinoma, there were no objective clinical responses, and *in vitro* evidence of vaccine-induced anti-CEA immune responses was found in only a minority of vaccinated patients. This suggests that additional immunostimulatory signals or adjuvants may be needed to break tolerance to human tumor antigens and to elicit effective antitumor immune responses.

One potential strategy to enhance the activity of DNA vaccines is to utilize cytokines to attract, differentiate and activate antigen-presenting cells (APC). Granulocyte/macrophage colony-stimulating factor (GM-CSF) may be a particularly effective adjuvant for cancer vaccine approaches (8). The mechanism of GM-CSF adjuvant activity appears to be mediated in part by chemo-attraction and activation of APC which in turn internalize, process and present tumor

antigens to lymphocytes (9, 10). GM-CSF has been shown in mice to preferentially expand myeloid dendritic cells (DC) and to enhance responses to vaccines by increasing both the number and the maturation state of local DC (11, 12). GM-CSF also enhances macrophage phagocytic activity, major histocompatibility class II molecule expression, antigen-processing activity and tumor cell cytotoxicity (8).

It has been shown that immunization of C57BL/6 mice with a plasmid encoding CEA efficiently induces CEA immune responses that are protective against challenge with mouse tumor cells expressing human CEA (13), and that co-delivery of mouse GM-CSF on a separate plasmid can enhance the immune response (14). Physical linkage of the expressed target antigen and GM-CSF *in situ* may more effectively target the uptake and presentation of antigen by GM-CSF-receptor expressing APC. Several chimeric fusion proteins containing GM-CSF have been reported which retain biological activity and exhibit enhanced immunogenicity of the tumor antigen (15-17). Here we show that fusion of CEA with GM-CSF in a plasmid vaccine elicits a more potent anti-CEA immune response in a mouse model of adenocarcinoma, but that the fusion construct can elicit potentially deleterious GM-CSF autoantibodies.

MATERIALS AND METHODS

Plasmid DNA Constructs

The mammalian expression plasmid pGT37 (18) encoding full length CEA was obtained from Dr. R. M. Conry and is referred to as pCEA(full length). The mouse GM-CSF plasmid (pNGVL1-mGMCSF) was obtained in the pNGVL1 backbone from the National Gene Vector Laboratories (NGVL, Ann Arbor, MI) and is referred to as pGMCSF. The GFP encoding plasmid, pCMS-EGFP, was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Additional plasmids were constructed in the pNGVL3 vector (NGVL). A short, secretory form of CEA, CEA(70), was obtained as a PCR product from the cell line MC38-CEA-2 (19) using the following primers: forward 5'-GTAAGTCGACGCGACCATGGAGTCTCCCTCGGCCCC-3' and reverse 5'-CCTTGAATTCC*AGAGCCTCCGCCACCTGAACCTGC*TGATGCAGAGA CTGTGATGCT-3', with *Sal*I and *Eco*RI sites underlined and reverse primer sequences encoding a flexible linker (amino acids AGSGGGGS) in italics. The MC38-CEA-2 cell line contains a spontaneous internal deletion within the three repetitive domains of CEA which creates a single chimeric repeat domain (19). The reverse primer was designed to amplify CEA sequences upstream of the carboxyl terminal GPI membrane-anchoring region, omitting the final 25 amino acid residues of CEA, so the product would be efficiently secreted. The amplified DNA fragment was cloned into *Sal*I/*Eco*RI sites of pNGVL3 to generate pCEA(70) which was used for immunization and for construction of fusion plasmids with CEA at the amino terminus. For fusion constructs encoding CEA at the carboxy terminus, CEA was amplified from MC38-CEA-2 cells using forward primer 5'-GGAAGGTACCA*GCAGGTTCAGGTGGCGGAGGCTCT*

AAGCTCACTATTGAATCCACGC-3'and reverse primer 5'-CCTTTCTAGATCAAGATGCA GAGACTGTGATGCTCTTG-3', with *Kpn*I and *Xba*I sites underlined and the flexible linker in italics, again removing the GPI anchor site, and the cDNA was cloned into *Kpn*I/*Xba*I sites of pNGVL3.

For construction of plasmids encoding mouse GM-CSF fused to the carboxyl terminus of CEA(70), GM-CSF was amplified from pGMCSF using forward primer 5'-GGAAGGTACCAGCACCCACCCGCTCACCCATC-3' and reverse primer 5'-CCTTTCTAGATCATTTTTGGCTTGGTTTTTTGCA-3' (*Kpn*I and *Xba*I sites underlined), and the fragment cloned into the *Kpn*I/*Xba*I sites of pCEA(70) to generate the plasmid called pCEA(70)-GMCSF. For fusion of GM-CSF at the amino terminus of CEA, GM-CSF was amplified using forward primer 5'-GGAAGTCGACATGTGGCTGCAGAATTTACTTTTC-3' and reverse primer 5'-GCCTGAATTCCTTTTTGGCTTGGTTTTTTGCATTC-3' (*Sal*I and *Eco*RI sites underlined) and cloned into the *SalI*/*Eco*RI site of the plasmid containing CEA with flexible linker at the amino terminus. All constructs were verified by complete DNA sequencing (Center for AIDS Research DNA Sequencing Core Facility, University of Alabama at Birmingham). Validated plasmid DNA constructs were prepared from Max Efficiency DH5α cells (Life Technologies, Inc., Rockville, MD) using Endotoxin Free Qiagen Plasmid Purification System (Qiagen Inc., Valencia, CA), according to manufacturer's directions.

Cell Lines

The murine adenocarcinoma cell line MC38-CEA-2 (19) was provided by Dr. Jeffrey Schlom, National Cancer Institute. C_2C_{12}, a mouse myoblast cell line, was obtained from the American Type Culture Collection (ATCC CRL-1772, Manassas, VA). These cell lines were

maintained in DMEM medium (Mediatech, Herndon, VA) containing 10% heat inactivated FCS (Hyclone, Logan, UT). The GM-CSF/IL-3 dependent murine bone marrow cell line, FDC-P1 (ATCC CRL-12103) was routinely cultured in DMEM containing 10% heat inactivated FCS, 4 mM L-glutamine (Mediatech) and 25% WEHI-3 (ATCC TIB-68) conditioned medium (20).

Mice

Female C57BL/6 mice 4-8 weeks of age were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) and were housed in the Pathogen-Free Rodent Shared Facility (Comprehensive Cancer Center, University of Alabama at Birmingham). All animal procedures were performed in accordance with recommendations for the proper care and use of laboratory animals.

Immunoprecipitation and Western Blot Analysis

To validate plasmid constructs, 1 μg of plasmid DNA was transfected into 60-80% confluent C_2C_{12} cultures in 12 well plates using 15 μl of Lipofectamine (Life Technologies) as recommended by the manufacturer. Forty-eight hours after transfection, 1 ml of media from transfected cultures was collected and subjected to immunoprecipitation with 5 μg/ml of biotinylated single-chain CEA antibody (21, kindly provided by Dr. M. B. Khazaeli) by incubation at 4°C overnight with rotation, followed by addition of 5 μg/ml of streptavidin-agarose beads (Sigma Chemical Co. Aldrich, St. Louis, MO) and further incubation at room temperature for 2 hr with rotation. Tubes were centrifuged at 1000 x g for 2 min, supernates discarded, and pellets washed once with PBS. Beads were resuspended in sample buffer (50 mM

Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and proteins separated by electrophoresis on a 10% polyacrylamide SDS gel. Proteins were electroblotted to nitrocellulose membranes (BioRad, Richmond, CA) for 1 hr at 4°C and 75 volts. Membranes were blocked with Tris-buffered saline (TBS) containing 2% nonfat milk for 2 hr at room temperature. Membranes were probed with either 2.5 μg/ml COL-1 mouse monoclonal antibody to CEA (NeoMarkers, Fremont, CA) or 5 μg/ml rat anti-mouse GM-CSF antibody (Pharmingen, San Diego, CA) in TBS/2% nonfat milk for 2 hr, washed 3 times with Tris-buffered saline plus 0.5% Tween 20 (TBST), and then incubated with alkaline phosphatase (AP) conjugated goat anti-mouse IgG or goat anti-rat IgG2 (Southern Biotechnology Associates Inc., Birmingham, AL) diluted 1:2000 in TBS/2% nonfat milk. Membranes were washed 3 times with TBST and developed using the AP Substrate Kit IV BCIP/NBT (Vector Laboratories, Inc., Burlingame, CA).

GM-CSF Functional Activity Assay

One μg of plasmid DNA was transfected into confluent C_2C_{12} cells as above, and culture medium was collected 48 hr later and concentrated 10-fold with a Centricon YM-10 filter (Millipore Corporation, Bedford, MA), with the final concentrate sterilized by filtration through 0.22 μm (Spin-X, Costar, Cambridge, MA). FDC-P1 cells were cultured for 48 hr in 96-well flat bottom plates at 5×10^4 cells/well, in the absence of WEHI-3 conditioned medium, with 50% (v/v) of the concentrated transfected cell supernates. Cultures were then pulsed with 1 μCi/well ^{3}H-thymidine (New England Nuclear, Boston, MA), and cultured for an additional 12 hr before harvest using a Micro 96 cell harvester (Skatron Instruments, Sterling, VA). Assay of incorporated radioactivity was performed with the Matrix 9600 Direct Beta Counter (Packard,

Downers Grove, IL). To assay for neutralization of GM-CSF function by serum antibodies, FDC-P1 cells were cultured as above in the presence of 2.5 ng/ml of recombinant mouse GM-CSF (Peprotech, Rocky Hill, NJ) with the addition of 0.22 μm filtered immune mouse sera at a final dilution of 1:12.

DNA Vaccination and Tumor Challenge

Two immunization protocols were used. In the high-dose protocol, mice received 50 μg of plasmid intramuscularly (i.m.) in the tongue 3 times, 3 weeks apart. Seventeen days after the last immunization, mice were challenged with 3×10^5 MC38-CEA-2 cells s.c. in the right flank. In the low-dose protocol, a single dose of 5 μg of plasmid was injected i.m., with tumor challenge 3 weeks later as above. Tumors were measured every 2-3 days and volume (cm^3) calculated by the formula $LxW^2/2$.

ELISA for Serum Antibodies

For CEA antibody detection, 96 well EIA plates (Costar 3590) were coated with human CEA protein (Fitzgerald Industries International, Inc., Concord, MA) at 1 μg/ml in borate saline (BS) buffer, pH 8.4, for 4 hr at room temperature, and then blocked with borate saline plus 1% (w/v) bovine serum albumin (BS-BSA). Serial three-fold dilutions of mouse serum in BS-BSA (1:50 - 1:109,350) were added to duplicate wells and incubated overnight at 4°C. Plates were washed with PBS + 0.05% (v/v) Tween-20 and incubated with either AP conjugated goat anti-mouse IgG, anti-IgM or anti-IgG isotypes γ1, γ2a, γ2b, γ3 (Southern Biotechnology) diluted 1:2000 in BS-BSA for 4 hr at room temperature. After washing, AP substrate (Sigma) in

diethanolamine buffer, pH 9.0, was added and incubated for 20 min at room temperature. Absorbance was measured at 405 nm on a VersaMax microplate reader using SoftMax Pro software (Molecular Devices, Sunnyvale, CA). Absorbance on CEA coated plates was corrected for absorbance on parallel plates coated with ovalbumin (Sigma). COL-1 mouse monoclonal γ2a antibody to CEA (NeoMarkers) was used as a positive control. For estimation of antibody isotype content, data were normalized to artificial controls using EIA wells coated with goat anti-mouse Ig (H+L) and subsequently incubated with purified mouse IgM, IgG1, IgG2a, IgG2b or IgG3 at known concentrations (Southern Biotechnology), followed by detection with the μ or γ isotype-specific antibody conjugates. To detect GM-CSF antibodies, sera were assayed as above on EIA plates coated with 1 μg/ml recombinant mouse GM-CSF (Peprotech) and subsequently incubated with AP conjugated goat anti-mouse IgG (Southern Biotechnology) followed by AP substrate and absorbance measurement.

Cytokine Release Assays

Single cell suspensions of splenocytes were prepared by mincing and forcing spleen tissue through a 100 μm sterile nylon strainer (Falcon 35-2360) in PBS. Erythrocytes were removed by hypotonic lysis and cells cultured in RPMI-1640 + 10% FCS, 4 mM L-glutamine and 12.5 μM β-mercaptoethanol at 1 x 10^5 cells/well in round bottom 96 well plates (Linbro 75-042-05). Cell were cultured in the presence of 25 μg/ml purified human CEA protein (Aspen Bioincorporated, Littleton, CO), or as negative controls, media alone or 50 μg/ml ovalbumin (Sigma). After 3 days, culture supernatants were collected and assayed for mouse IFN-γ and IL-

4 by ELISA kits (Biosource International, Camarillo, CA) according to the manufacturer's instructions.

RESULTS

Construction and Validation of Fusion Plasmids

Two plasmids encoding fusions between CEA(70) and mouse GM-CSF were designed, with mouse GM-CSF placed either on the carboxyl [pCEA(70)-GMCSF] or amino [pGMCSF-CEA(70)] terminus of CEA, and the fusion components separated by a glycine/serine-rich flexible linker. To allow flexibility in the construction of genetic fusion proteins, we cloned a short version of CEA, CEA(70), derived from the MC38-CEA-2 cell line, which contains a spontaneously arising deletion within the homologous internal repeat domains of CEA (19). CEA(70) was additionally modified to remove the GPI membrane anchor region so that the encoded protein would be efficiently secreted. The natural signal peptide sequence of the amino-terminal moiety (CEA or GM-CSF) was preserved.

To validate expression and secretion of the encoded fusion proteins, supernatants from C_2C_{12} cells transfected with the two fusion plasmids or control plasmids were evaluated by immunoprecipitation and Western blot analysis. As shown in Figure 1A, proteins of the expected size were produced, secreted and appropriately recognized by antibodies specific for CEA and mouse GM-CSF. The GM-CSF portion of both plasmid fusion proteins was biologically active as determined by the ability to support growth of the GM-CSF dependent cell line FDC-P1 *in vitro* (Fig. 1B).

High-Dose Plasmid Immunization

We first tested plasmid immunization using a high-dose schedule. Mice were immunized 3 times with 50 μg plasmid DNA every 3 weeks. Sera from immunized mice were assayed for CEA antibodies 2 weeks following the first and the third immunizations. None of the control groups (naïve, empty vector or pGM-CSF alone) had detectable antibodies against CEA. In contrast, all CEA containing plasmids elicited IgG antibody responses to CEA by day 14 after the first immunization (data not shown), with higher titers observed on day 56, two weeks after the third immunization (Fig. 2A). Isotype analysis of CEA antibodies at day 56 (Fig. 3A) demonstrated a similar pattern of responses for all CEA-containing plasmids, with the predominant isotype being IgG1, and detectable IgG2a and IgG2b, but no IgG3 or IgM antibodies to CEA.

To evaluate CEA-specific T cell activation, spleen cells from day 56 of immunization were stimulated *in vitro* with human purified CEA protein and culture supernatants assayed for IFN-γ and IL-4 release by ELISA. Spleen cells from mice immunized with all CEA containing plasmids showed CEA-specific IFN-γ release (Fig. 4), but no antigen-specific IL-4 release was detected (data not shown). Together with the antibody isotype analysis, these data are suggestive of a Th1 type immune response in mice immunized with CEA-encoding plasmids.

Mice were challenged with syngeneic MC38-CEA-2 cells 17 days following the third immunization, and tumor growth monitored (Fig. 5A). All three control groups demonstrated 100% progressive tumor growth by day 21 after challenge. In contrast, all mice immunized with

CEA-expressing plasmids (including fusion plasmids) were tumor free until day 38 following challenge. After this time, late onset tumor growth was observed in 60-70% of the mice injected with either of the GM-CSF-CEA fusion plasmids, whereas 90% of the mice immunized with pCEA(70) and 100% of the animals vaccinated with pCEA(full length) were tumor free at day 60.

GM-CSF recombinant proteins have been reported to induce autoantibodies, which in turn might decrease the effectiveness of vaccine immune response (16, 17, 22). We therefore evaluated serum antibody to recombinant mouse GM-CSF by ELISA. As shown in Fig. 6A, sera from control immunization groups showed no detectable GM-CSF reactivity, whereas sera from mice vaccinated with the pCEA(70)-GMCSF fusion had significant titers of IgG reactive with GM-CSF; mice immunized with the pGMCSF-CEA(70) fusion construct showed similar levels of such autoantibodies (data not shown). Of note, immunization with plasmid encoding mouse GM-CSF alone (pGM-CSF) did not elicit detectable autoantibodies (Fig. 6A). To test whether the elicited GM-CSF antibodies could neutralize cytokine activity, FDC-P1 cells were cultured with recombinant mouse GM-CSF in the presence of sera from vaccinated mice (Fig. 6B). Inhibition of FDC-P1 growth was observed in the presence of sera from mice immunized with both GM-CSF-CEA fusion plasmids, but not in the other tested sera, indicating that autoantibodies induced by fusion plasmid immunization could specifically neutralize GM-CSF biologic activity.

Low-Dose Plasmid Immunization

We next tested the effectiveness of a low-dose plasmid immunization schedule, which we had previously determined to protect approximately 50% of mice immunized with plasmid

encoding full length CEA from MC38-CEA-2 tumor challenge. Mice received a single injection of only 5 μg of plasmid vaccines, a 10-fold lower dose than that used for each of the 3 immunizations of the high-dose protocol. On day 10 following the single low-dose immunization, no antibodies against CEA were detected in any of the injected mice (data not shown). By day 20, serum IgG antibodies to CEA were detected in all groups vaccinated with CEA containing plasmids, but not in the control immunization groups (Fig. 2B). CEA antibody titers obtained with low-dose immunization were substantially lower than those obtained with the high-dose schedule (Fig. 2A). For low-dose immunization with plasmids encoding CEA alone, CEA-specific antibody IgG isotypes profiles were similar to those observed following high dose immunization. However, mice immunized with the low-dose schedule of CEA-GM-CSF fusion plasmids exhibited lower proportions of IgG2a and IgG2b serum antibodies to CEA as compared to the respective high dose immunization sera (Fig. 3). No IgM antibodies to CEA were detected in any studied sera. Cytokine release assays using spleen cell cultures as described above for the high-dose immunization failed to produce any detectable IFN-γ or IL-4 in the low-dose immunized mouse cells cultured with CEA protein (data not shown).

GM-CSF autoantibody titers were substantially lower following low-dose as compared to high-dose immunization. Again, IgG autoantibodies were detected only in mice vaccinated with the fusion constructs, and not in mice immunized with pGMCSF alone or with pGMCSF co-administered with pCEA(70) (Table 1). Neutralization assays using the GM-CSF-dependent FDC-P1 cell line and recombinant mouse GM-CSF failed to demonstrate neutralizing activity in sera of mice immunized with low-dose plasmid fusion proteins (data not shown).

Figure 5B presents MC38-CEA-2 tumor challenge data from mice immunized with the low-dose schedule, combining data from two independent experiment. Control mice immunized

with pGMCSF alone developed tumors by day 25 following challenge. In contrast, 60% of mice immunized with pCEA(full length) and 50% of mice immunized with pCEA(70) were tumor free at the end of the experiment. Both GM-CSF fusion plasmids afforded a greater percent protection than either CEA plasmid alone: 80% of mice immunized with pGMCSF-CEA(70) remained tumor free at day 90 [$p \leq 0.05$ by comparison to pCEA(70) alone], and 65% of mice immunized with pCEA(70)-GMCSF were protected (not statistically significant compared to CEA plasmid alone). Co-injection of pGMCSF and pCEA(70) as separate plasmids also afforded 65% protection against tumor challenge. In contrast to results following high-dose immunization (Fig. 5A), mice immunized with the CEA-GM-CSF fusion plasmids by the low-dose schedule did not demonstrate excess late-stage tumor development as compared to mice immunized with CEA plasmids alone (Fig. 5B).

DISCUSSION

DNA-based immunization may offer significant advantages compared to other forms of immunization for cancer immunotherapy applications (1). Importantly, the intracellular synthesis of the target antigen by host cells following injection of plasmid vaccines may promote the induction of a Th1-associated cellular immune response that is critical to the development of effective antitumor immunity. Many tumor-associated antigens are self-antigens to which there is a high degree of tolerance, and eliciting cellular immune response to self-antigens represents a considerable challenge for tumor vaccines.

A number of strategies have been evaluated to augment the potency of immune response induction by DNA-based immunization. The co-injection of plasmids encoding several cytokines has been shown to enhance the cellular immune response, humoral immune response, or both (23). Among these, GM-CSF is attractive due to its ability to recruit APC to the site of antigen synthesis and its ability to stimulate maturation of DCs (8). The co-injection of GM-CSF plasmids has been effective in enhancing the activity of DNA immunogens in several, but not all, preclinical studies (14, 24-28). Fusion constructs consisting of the target antigen linked to GM-CSF have been tested by several groups, with the goal of enhancing immunogenicity as well as targeting the fusion proteins for uptake by APC expressing GM-CSF receptors (15-17).

Previous studies in the CEA mouse model showed enhanced antitumor responses with coinjection of plasmids encoding the human CEA tumor antigen and GM-CSF (14). In the current study, we examined the immunologic and anti-tumor activity of a DNA vaccine encoding

CEA fused with GM-CSF. The results indicate that fusion constructs were capable of eliciting critical Th1-asssociated cellular immune responses as evidenced by antibody isotype induced and the production of IFN-γ in response to CEA. More importantly, this immune response was associated with antitumor activity.

A potential drawback to the clinical use of GM-CSF-CEA fusion construct is the induction of GM-CSF autoimmune responses that may interfere with antitumor immune response. Immunizing mice with plasmids expressing cytokines or chemokines has resulted in autoantibodies capable of modulating immune activity in mouse studies (29, 30). More specifically, GM-CSF autoimmunity induction has been reported in several clinical studies. Immunizations of cancer patients with recombinant GM-CSF protein mixed with tumor antigen (22), with an idiotype-GM-CSF fusion protein (16), or with DC pulsed with a GM-CSF protein fusion to prostatic acid phosphatase (17) have been reported to elicit cellular and/or humoral immune response to GM-CSF. In addition, administering the GM-CSF/IL-3 fusion protein PIXY 321 has also elicited anti-GM-CSF antibody in cancer patients (31). To date, there has been no evidence of clinical complication from such GM-CSF specific autoimmunity.

In our study, only plasmids encoding protein fusions between GM-CSF and CEA elicited detectable immune responses to GM-CSF (Table 1, Fig. 6A). At least some of the induced anti-GM-CSF antibodies had cytokine neutralizing activity *in vitro* (Fig. 6B), suggesting that this may have suppressed the vaccine induced anti-CEA immune response and contributed to the late growth of tumors following the high-dose immunization schedule (Fig. 5A). Interestingly, our *in vitro* assays of immune response (*e.g.*, Figs. 2 and 3) did not reveal correlative quantitative or qualitative differences between CEA immune responses induced by CEA vaccine inducing GM-CSF autoantibodies as compared to those that did not.

It can be speculated that because CEA is a foreign antigen in these mice, CEA may have acted as a "carrier" to induce immune responses to the fused GM-CSF polypeptide. There is no homologue of human CEA in mice, which may accentuate this hypothesized carrier effect. Alternatively, the structure of the GM-CSF in the context of the fusion protein may have been altered in a way to promote its activation of the immune system. In the 38C13 murine lymphoma, low-dose administration of GM-CSF was more effective in potentiating vaccine responses than a high-dose (32). Tumor cells transduced with GM-CSF were also more effective in stimulating immunity in other murine models if they were low-producers as compared to high-producers of GM-CSF (33). The data presented here suggest that a lower dose of the GMCSF-CEA(70) plasmid was better able to protect animals from challenge with CEA-expressing tumors, as directly compared to parallel immunization with CEA plasmids alone. Although autoantibodies to GM-CSF were still detectable, they were of lower titer than those induced with the high dose immunization schedule, and no cytokine neutralizing activity was detected. Of note, immunization with plasmid DNA encoding mGM-CSF alone did not induce detectable GM-CSF antibodies even when co-administered with plasmids encoding CEA alone (Table 1).

CEA is an attractive target for cancer vaccine approaches. This 180 kd glycosylated protein is displayed on the cell surface via a GPI membrane anchor and is released into the circulation via mechanisms apparently common to such GPI anchors (34, 35). In this study we elected to construct GM-CSF-CEA fusion plasmids using a shortened form of CEA that was further modified to remove the GPI anchor so that it would be efficiently secreted. The rationale for this modifications was as follows: First, the shortened pCEA(70) protein should be ~100 kd smaller than full length CEA, and thus plasmid encoded fusions of CEA(70) with molecules such as GM-CSF might be more efficiently expressed *in vivo* due to the significant reduction in fusion

protein size. Second, efficient release of the CEA(70)-GMCSF fusion proteins from transduced cells *in vivo* would be necessary to engage GM-CSF receptors on large numbers of APC, for efficient internalization of CEA(70) protein for APC processing and presentation to lymphocytes. Although not statistically significant, there was a trend in the tumor protection data suggesting that pCEA(70) immunization was less effective than that of the plasmid encoding full length CEA. This might be due to several factors, including (a) the different plasmid backbones of pCEA(full length) and the truncated CEA constructs, (b) expression of a secreted CEA versus the membrane-associated CEA, or (c) loss of immunogenic domains in the pCEA(70) internal deletion. Studies to address these possibilities are currently underway.

In summary, the incorporation of GM-CSF into DNA-based cancer immunotherapy protocols targeting CEA may enhance efficacy. The GMCSF-CEA(70) fusion construct described here can elicit Th1-associated cellular immune responses to CEA and elicit a protective antitumor immune responses in mice. Enhanced anti-tumor efficacy is dose and schedule dependent. However, as this study indicates, plasmid encoded fusions between cytokines and tumor antigens can break immune tolerance to the cytokine moiety. In the worst case this may have long-term detrimental effects for the immunized host, and at minimum it could undermine the desired cytokine immune adjuvant effects. Careful control of GM-CSF dose and appropriate monitoring will be necessary to avoid a potentially detrimental GM-CSF autoimmune responses.

FIGURE LEGENDS

Figure 1. Validation of plasmid-encoded fusion protein secretion and function. (A) Supernates from C_2C_{12} cells transfected with the indicated plasmids were subjected to immunoprecipitation with CEA antibody, followed by SDS-PAGE and transfer to nitrocellulose membranes. Proteins were detected with either mouse anti-human CEA (right) or rat anti-mouse GM-CSF (left). Supernates from cells transfected with plasmids encoding green fluorescent protein (pGFP) or mouse GM-CSF (pGMCSF) served as negative controls. (B) Cytokine activity of GM-CSF fusion proteins was assessed by proliferation of FDC-P1 cells, measured by ^{3}H-thymidine uptake, following incubation of cells with supernates from C_2C_{12} cells transfected with the indicated plasmids. Data are mean incorporated cpm for quadruplicate samples, with error bars showing SD of the mean.

Figure 2. CEA antibody titers in sera from DNA immunized mice. Pools of sera (n=12) from each group collected after immunization were assayed by ELISA for reactivity with purified human CEA. Each dilution was tested in duplicate, and mean absorbance on plates coated with CEA was corrected for absorbance on parallel plates coated with ovalbumin. (A) High-dose immunization sera collected on immunization day 56, 14 days after the third 50 μg vaccine dose. (B) Low-dose immunization sera collected on day 20 following a single 5 μg vaccine dose.

Figure 3. CEA antibody isotypes in sera from plasmid immunized mice. Pools of sera (n=12) from each immunization group as in Fig. 2 were assayed by ELISA on CEA-coated plates and

developed with AP conjugated antibodies specific for the indicated mouse immunoglobulin heavy chain isotypes. Data represent mean absorbance of duplicates at a single serum dilution relative to absorbance of the 5 μg/ml mouse isotype artificial standard. (A) High-dose immunization sera as in Fig. 2 tested at a 1:450 dilution. (B) Low-dose immunization sera as in Fig. 2 tested as a 1:50 dilution.

Figure 4. CEA-specific IFN-γ release by mouse spleen cells following high-dose DNA immunization. Pooled splenocytes (n=2) collected on day 56 of the high-dose immunization schedule were cultured with either CEA, ovalbumin or medium alone for 3 days. Culture supernates were collected and assayed for IFN-γ by ELISA. Data represent mean IFN-γ concentrations of duplicate samples, with error bars showing SD of the means.

Figure 5. Tumor-free survival of mice immunized with DNA vaccines. Immunized mice were challenged s.c. with MC38-CEA-2 cells and tumor development assessed every 2-3 days. Data are presented as percent tumor-free survival. (A) High-dose immunization mice (n = 10) were challenged with tumor cells 17 d following the third vaccination with 50 μg of the indicated plasmids. (B) Low-dose immunization mice (n = 20, except for the pCEA(70)-GMCSF group where n = 19) were challenged with tumor cells 21 d following a single vaccination with 5 μg of the indicated plasmids.

Figure 6. GM-CSF autoantibodies following immunization with fusion plasmids. (A) Pools of sera (n=12) from high-dose immunization groups as in Fig. 2 were assayed by ELISA on plates coated with recombinant mouse GM-CSF in duplicate, and the mean absorbance corrected for

absorbance on parallel plates coated with ovalbumin. (B) FDC-P1 cells were cultured in the presence of recombinant mouse GM-CSF, in the presence of day 56 pooled sera (1:12 final dilution) from the indicated high-dose immunized mice. After 3 days, FDC-P1 cell proliferation was measured by ^{3}H-thymidine uptake. Data are mean incorporated cpm of quadruplicate samples, with error bars indicating SD of the means.

Tables

Table 1. Autoantibodies to mouse GM-CSF following DNA immunization.

serum source	mean Abs 405 nm anti-IgG	anti-IgM
High dose immunization		
pCEA(70)-GMCSF	1.40	0.04
pGMCSF-CEA(70)	2.01	0.06
Low dose immunization		
Naïve	0.01	0.04
pCEA(full length)	0.00	0.05
pGMCSF	0.00	0.03
pCEA(70)	0.00	0.04
pCEA(70) + pGMCSF	0.01	0.04
pCEA(70)-GMCSF	0.18	0.05
pGMCSF-CEA(70)	0.33	0.04

Pools of sera (n=12) from each immunization group at a final dilution of 1:100 were assayed for IgG and IgM antibodies to recombinant mouse GM-CSF by ELISA. High-dose sera were from day 56 following three immunizations with 50 μg, and low-dose sera were from day 20 following a single immunization with 5 μg. Data are mean absorbances of duplicate samples.

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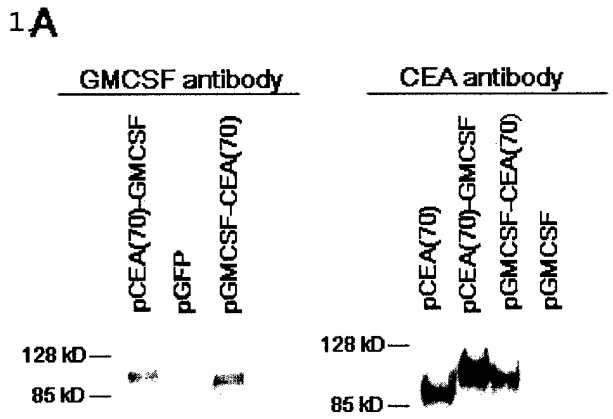

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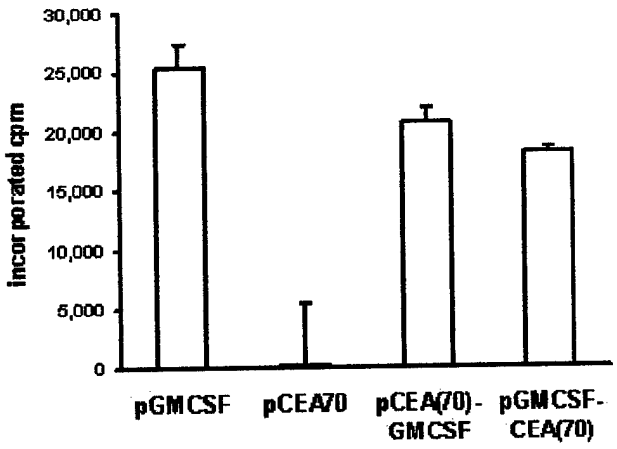

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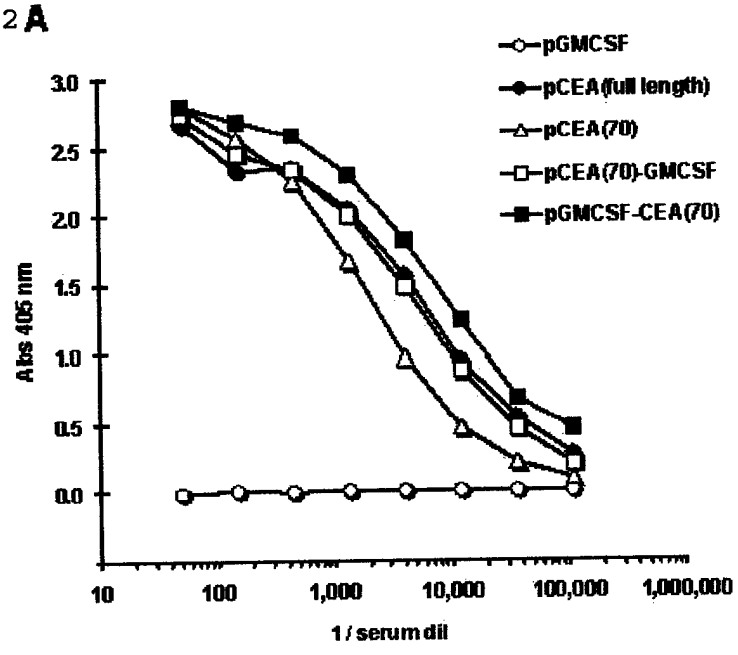

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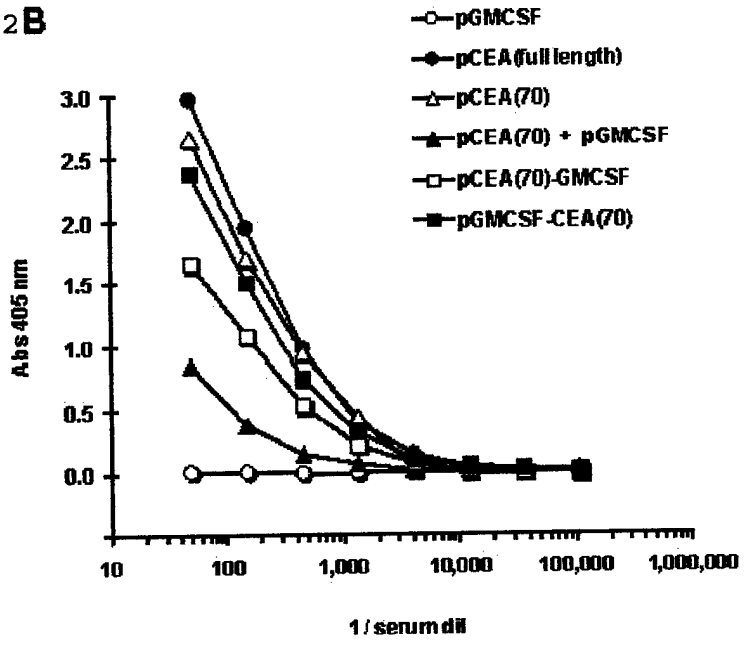

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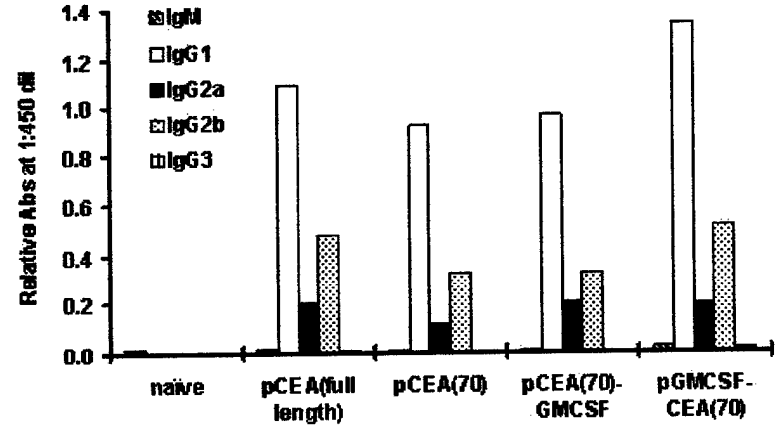

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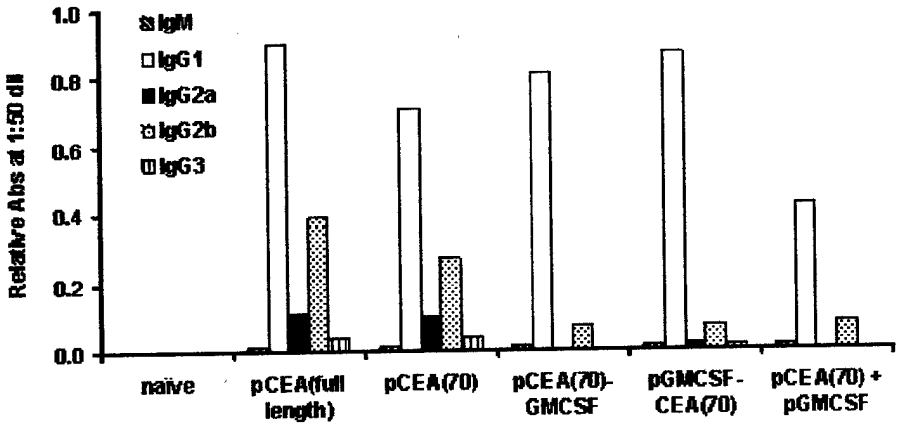

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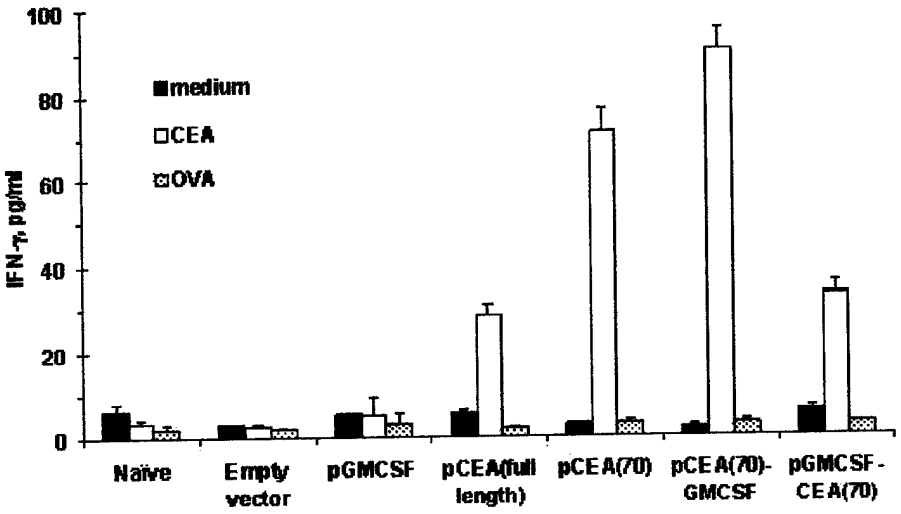

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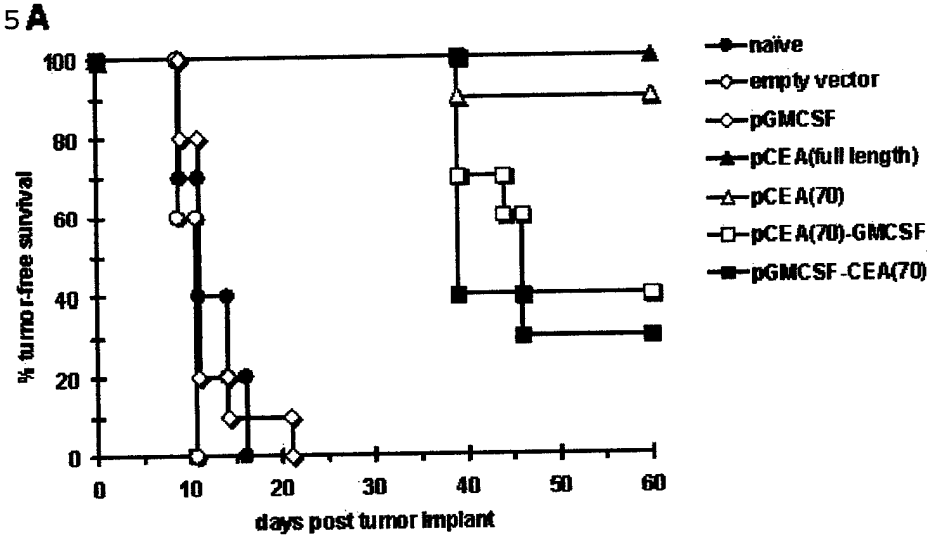

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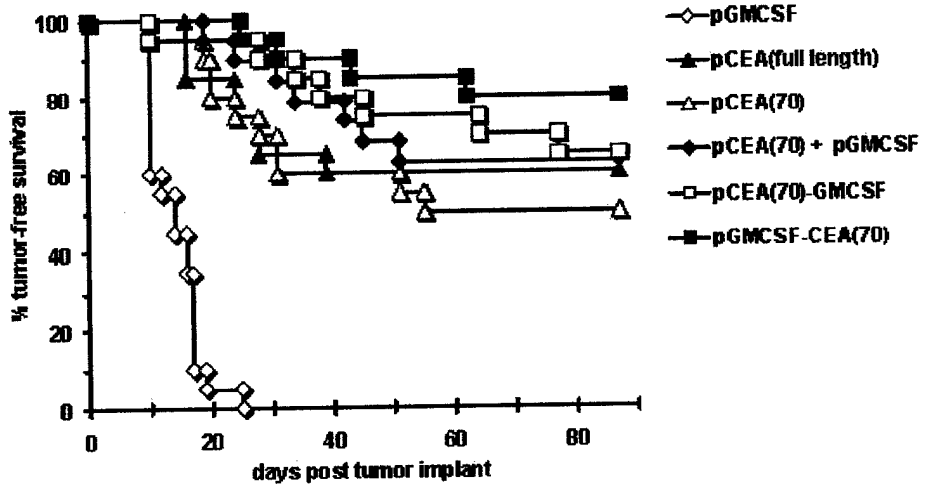

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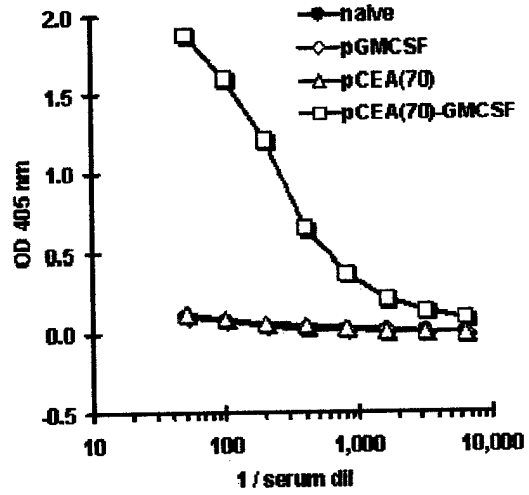

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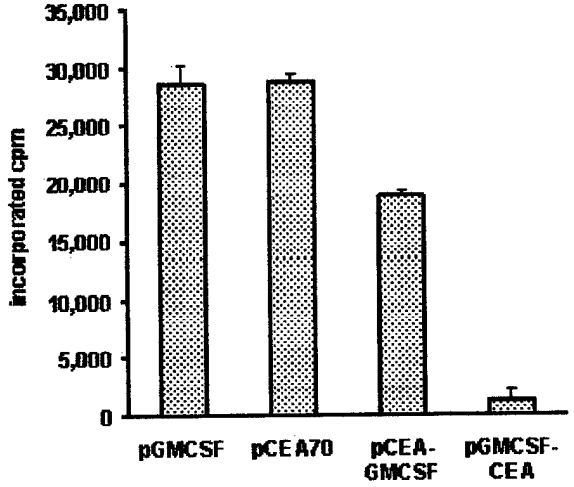

CANCER GENE THERAPY

Curiel and Douglas, eds.

Polynucleotide Immunization for Cancer Therapy

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1. INTRODUCTION

The limitations of conventional cancer therapy (surgery, radiation and chemotherapy) combined with an improved understanding of the molecular mechanisms regulating the immune system have led to increasing attention focused on the development of immunotherapies for cancer. Active immunotherapy approaches seek to eliminate tumor cells by eliciting immune responses directed against tumor associated antigens. Gene transfer techniques have expanded the potential opportunities in this area by providing new methods for stimulating the immune response. Among the array of techniques being developed for clinical application, nucleic acid or polynucleotide vaccines have emerged as a novel and effective method of inducing tumor antigen-specific immune responses.

Rather than immunizing with a protein, polynucleotide immunization (PNI) relies on delivery of DNA or RNA molecules encoding an antigen of interest. There are several advantages to this mode of delivery. Perhaps most importantly, both antibody and cellular immune responses are elicited following PNI. The *in vivo* synthesis of the encoded antigen allows the protein to be processed for presentation on the major histocompatibility class (MHC) class I complex, promoting the generation of class I restricted cytolytic T lymphocytes (CTLs). Because CTLs are known to be important mediators of the antitumor immune response, their activation against tumor antigens is critical to the success of cancer vaccine approaches. Furthermore, and in contrast to protein vaccines prepared in nonmammalian hosts, synthesis of the antigen *in vivo* allows appropriate folding and post-translational modification of the protein. DNA based vaccines also maintain antigen expression for extended periods, supporting persistent antitumor immune responses that should theoretically protect a patient from relapse. Additional factors favoring the development of plasmid DNA-based immunization strategies include the relative ease and inexpensive nature of vaccine preparation, as well as its stability. As discussed in more detail below, DNA vaccines prepared in bacterial hosts are inherently immunostimulatory due to the presence of unmethylated CpG dinulcoetides. These sequences stimulate a nonspecific immune response that does not interfere with repeat delivery of the vaccine. This contrasts with viral-based vaccines, where pre-existing or vector induced immune responses can strongly compromise the effectiveness of the vaccine [1] [2]. Safety considerations also favor polynucleotide vaccines compared to viral vaccines, since there is no risk for recombination with wild type viruses and the risk of insertional mutagenesis is quite low. Finally, DNA and RNA vaccines

have the potential to readily deliver multiple epitopes, and even multiple antigens, in a single injection; an important consideration given the propensity of tumors to escape immune detection by antigen loss variants [3].

Despite these potential advantages and encouraging preclinical studies, polynucleotide vaccines for cancer have thus far shown only minimal activity in the clinical setting. Many tumor antigens are not mutated, and therefore induction of an immune response to these antigens requires that the immune system be able to recognize and mount an effective response to a 'self' antigen. Initial studies suggest that this will be difficult to achieve in the setting of human cancer. Therefore, improving the potency, and thereby the clinical efficacy, of polynucleotide vaccines has become the major focus of research in the field. This chapter will delineate some of the approaches, currently under evaluation in preclinical models, designed to address this limitation.

The versatility of DNA and RNA-based vaccines has led to the development of a number of delivery approaches to accomplish cancer immunotherapy. Whereas DNA or mRNA can be used to modify cells *ex vivo,* as in the case of transfected dendritic cells or irradiated tumor cells for vaccine therapy, this chapter will focus specifically on *in vivo* delivery of DNA or RNA for cancer immunotherapy.

2. GENE TRANSFER OF NUCLEIC ACIDS FOR IMMUNIZATION

The development of nucleic acid vaccines was sparked by the observation by Wolff and colleagues that intramuscular injection of naked DNA led to the expression of the encoded gene by myofiber cells[4]. Subsequent studies demonstrated the general applicability of this approach for the expression of foreign genes in a variety of species from fish[5] to nonhuman primates[6]. Although an inefficient process, the transferred DNA appears to enter the myofibers via the myocyte caveolae and T tubules [7] [8]. The DNA is maintained in an extrachromosomal form in the nucleus, but expression can be detected for a prolonged period[9], depending on the immunogenicity of the encoded protein. Ulmer and coworkers first demonstrated the ability of intramuscular delivery of DNA encoding a viral antigen to elicit a CD8+ T cell, MHC class I-restricted immune response protective against infection, using a plasmid encoding the influenza protein nucleoprotein A[10]. This study provided the rationale to develop polynucleotide vaccines for therapy of diseases, including cancer, previously not amenable to

traditional vaccine approaches that rely primarily on humoral immune responses. Rather than preventing disease, therapeutic immunization against chronic disease became a possibility.

Induction of cellular and humoral immune responses following delivery of nucleic acids is not limited to intramuscular delivery. The skin is rich in antigen presenting cells (APCs) such as immature Langerhans cells in the epidermis, and mature dendritic cells (DCs) in the dermis. Tang and co-workers demonstrated the ability of DNA delivered to the skin to elicit a humoral immune response to the encoded gene[11]. In this method, the DNA is delivered following precipitation onto gold microparticles[12]. The gold particles are delivered to the skin under pressure by a ballistic delivery device. The process, commonly referred to as gene gun delivery, does not produce traumatic injury and requires much less DNA to achieve comparable humoral immune response to intramuscular delivery [13] [14]. Induction of effector CTLs capable of mediating tumor rejection were subsequently demonstrated in a mouse model of transplantable tumors[15]. Intradermal immunization can also be accomplished by injection of naked DNA or by a needle-free jet injection system delivering DNA-coated nanoparticles[16]. Mucosal administration of DNA vaccines has also been explored primarily for immunization against infectious disease[17], but may also be applicable for cancer therapy[18]. In addition to naked DNA delivery to mucosal surfaces, plasmids can be delivered orally by employing bacteria as carriers, including attenuated *Salmonella* [19] [20] [21], *Shigella*[22], or *Listeria*[23] strains. Administration via bacteria may also contribute to the effectiveness of the vaccine by stimulating the innate immune response. Finally, despite a relatively short half-life in the circulation, studies on the intrasplenic administration of a DNA vaccine[24] demonstrated that strategies to promote uptake of DNA by splenocytes following intravenous administration might lead to induction of humoral and cellular immune responses. The fact that all of these delivery routes results in antigen synthesis and induction of antigen-specific immune responses attests to the flexibility of PNI. It is important to note that these different routes of administration may lead to qualitatively different immune responses[25] [26], and the relative efficacy in humans remains to be determined.

Although typically composed of plasmid DNA encoding a defined tumor antigen, vaccine strategies using mRNA have also been developed. One potential disadvantage of RNA-based vaccines compared to DNA vaccines is the considerably shorter half-life of mRNA. Nevertheless, the use of mRNA *in vivo* may be advantageous for immunization against oncogenic antigens such as the HER-2/neu gene, as the theoretical risk of transgene integration into the genome is eliminated. Qiu et al [27] used gene gun

delivery of mRNA to demonstrate expression of the encoded genes in the mouse epidermis and induction of antigen-specific antibodies. Intramuscular delivery of mRNA encoding carcinoembryonic antigen (CEA) also led to detectable anti-CEA antibodies and partial protection against challenge with CEA expressing tumor cells[28]. The immune response elicited by mRNA-based vaccines is generally of lower magnitude than DNA-based strategies, presumably due to the instability of the mRNA following delivery. To address this limitation, the use of self-replicating RNAs has emerged as a means to augment the efficacy of RNA-based immunization [29] [30] [31]. These vectors incorporate sequences into the RNA transcript that encode the RNA replicase polyprotein derived from Alphaviruses (e.g., Sindbis or Semliki Forest Virus). The replicase activity directs cytoplasmic replication of the entire transcript and also transcribes mRNA for the antigen from a subgenomic promoter, resulting in high levels of antigen expression. Further adaptation of the replicative RNA system has led to the development of plasmid DNA vectors encoding replicative RNA transcripts[32], which combine the ease of plasmid DNA vaccine preparation with the advantages afforded by replicative RNAs.

3. Mechanism of Immune Response Induction following Polynucleotide Immunization

The ability of polynucleotide vaccines to elicit a cellular immune response paved the way for their development as a reagent for cancer immunotherapy. The mechanism(s) for induction of the immune response following immunization is still not entirely clear, but appears to involve processing of the antigen through both endogenous and exogenous pathways leading to presentation of the antigen in the context of both MHC class I and class II. DNA may transfect both target cells (for example, myocytes after intramuscular delivery) as well as resident APCs. Although myocytes clearly synthesize the encoded protein, it is thought that only APCs are capable of delivering the costimulatory signals necessary to effectively prime CTLs. A number of studies support the central role for bone marrow-derived APCs in induction of the immune response following DNA immunization [33] [34] [35] [36]. The findings suggest a 'cross-priming' scenario, in which myocytes produce the antigenic protein and transfer it to APCs in such a way that the antigen is presented to T cells in the context of MHC class I, thereby allowing the APC to directly activate CTLs. Whereas proteins acquired exogenously by APCs are usually trafficked into the endolysosomal pathway for degradation and presentation by MHC class II molecules, in the case of PNI the processed antigen is available for both class I and class II presentation, promoting both cellular and humoral immunity.

Alternatively, or more likely in addition, the APCs themselves may be transfected by the transferred nucleic acid[37] [38]. The *in vivo* synthesis of the antigen in the cytoplasm promotes presentation of the peptide by MHC class I molecules. Proteins synthesized within the cell are endogenously processed into peptides by the proteasome. These peptides are loaded onto MHC class I molecules in the endoplasmic reticulum, and transported to the cell surface. Presentation of the antigen in the context of both class I and class II MHC, and in the presence of the appropriate co-stimulatory molecules, leads to the activation of both CD4+ and CD8+ T cells. The importance of the CD8+ CTL in mediating tumor cell destruction by recognition of antigenic peptide presented in MHC class I on the tumor cell surface is well established. These cells are known to play an important role in tumor cell destruction and long-term protection against rechallenge. The importance of the CD4+ cell in antitumor immunity has gained appreciation [39] [40]. These CD4+ T cells provide help for the induction of specific CD8+ CTL and secrete cytokines promoting the activation of CTL. In addition, they may activate nonspecific immune effector cells such as macrophages and eosinophils, which may further potentiate the destruction of tumor cells.

4. Factors Influencing Induction of Immune Response

A number of features of polynucleotide vaccines influence the nature and potency of the attendant immune response; from the composition of the nucleic acid itself, to the encoded antigen, to the microenvironment in which the vaccine is expressed. The composition of the DNA is an initial consideration for plasmid-based vaccines. The dinucleotide CpG is relatively underrepresented in the mammalian genome. Further, areas rich in CpG are frequently methylated as a mechanism of transcriptional regulation. In contrast to this, preparation of DNA vaccines in bacterial hosts results in the presence of unmethylated CpG dinucleotides in the plasmid. These unmethylated sequence are recognized by the innate immune system as indicative of the presence of a pathogen, and are immunostimulatory[41]. Specifically, the sequences are recognized by the toll-like receptor 9, and trigger activation of the innate immune system including DCs, macrophages, natural killer (NK) cells and NKT cells[42] [43]. The result is that CpGs, either present in the plasmid or delivered as purified oligodeoxynucleotides (ODN), are a potent adjuvant, biasing the immune system towards a Th1 type response[44]. The CpG-ODN also have an antiapoptotic effect on both CD4+ and CD8+ T cells, thereby expanding the pool of T cells and augmenting the immune response in an antigen-independent manner[45]. The presence of these CpG motifs contributes significantly to the overall immunogenicity of DNA vaccines.

Polynucleotide vaccines based on the RNA alphaviruses also are inherently immunostimulatory. These vaccines promote apoptosis in transfected cells, which may enhance immunogenicity[46]. The presence of a double-stranded RNA intermediate, which is generated during replication in the cytoplasm, activates the double-stranded protein kinase R (PKR) [47] and NF-kappa B[48], stimulating an innate antiviral pathway and thereby augmenting the immune response. An advantage of polynucleotide vaccines compared to viral vaccines is that the immunostimulatory pathways stimulated are nonspecific in nature, and do not interfere with readministration of the vector.

In addition to the composition of the nucleic acids, an important determinant of immune response is level of transgene expression. In general, increased immunogen expression augments the immune response. Hence a strong promoter is required to direct efficient transcription of the encoded protein, and optimized polyadenylation signals and untranslated regions may contribute to enhanced transgene expression[49]. The cytomegalovirus early promoter/enhancer has been widely used to drive expression of the encoded sequences, and may be enhanced by the insertion of additional sequences, for example those derived from the adeno-associated virus[50].

Once an optimized vector has been developed, the route of administration may also influence the resulting immune response. As discussed in the previous section, a number of routes of PNI have led to induction of cellular and humoral immune responses, but the nature of the immune response elicited by different routes of administration may be qualitatively different[25 26 51 52 53]. In general, gene gun administration of DNA leads to a more Th2-like immune response, with a strong humoral component that may be less effective for cancer therapy. However, this effect can be modified by co-administration of Th1 promoting cytokines[54]. The nature of the immune response can be further influenced by the vaccination dose and the schedule of administration[13 55].

The antigenicity of the encoded protein is of considerable importance in generating an effective response. The fact that most tumor antigens are 'self' represents a formidable challenge for all forms of active immunotherapy that rely on breaking immunological tolerance. Modifying the antigenicity of the protein or promoting its uptake by profession APCs are key areas of consideration in this respect. The local cytokine milieu also plays an important role in the immune response ultimately elicited.

Optimizing all of these factors so as to maximize the effectiveness of the immune response following PNI has become a major focus for investigators in this area of research.

5. Strategies to Enhance the Immune Response

DNA vaccines have shown promise in eliciting effective CTL responses to neoantigens, but the weakly immunogenic antigens characteristic of most tumors will require polynucleotide vaccines to be more potent if they are to be clinically useful. Thus, many studies have focused on enhancing the immune response elicited by PNI. Approaches have focused on every aspect of the vaccine, from delivery of the nucleic acid, to modification of the encoded antigen, to the perturbation of the microenvironment to maximize and tailor the immune response to a Th1 type response (Table 1). The versatility of polynucleotide vaccines is a strength in this respect, as both the nucleic acid and the encoded antigen of interest can be readily manipulated and evaluated.

Because the process of delivery of the nucleic acid to target cells is inefficient, approaches to increase the efficacy of delivery and/or increase the stability of the nucleic acid *in vivo* can result in higher and extended expression of the encoded antigen, increasing the magnitude of the immune response. To this end, incorporation of the nucleic acid into liposomes may protect it from endogenous nucleases and also promote uptake into cells[56]. Adsorption of DNA onto the cationic microparticles composed of poly(DL-lactide-co-glycolide) (PLG) allows the slow release of the DNA and results in a more potent immune response compared to naked DNA[57]. To physically enhance the transport of nucleic acids into the target cells, electroporation into skin[58] or muscle[59] has proven an effective means of increasing gene transfer efficiency. Application of this technology in the clinical setting will require careful optimization in human subjects.

The ease of manipulation of recombinant cDNAs allows the encoded antigen to be readily altered in ways that may enhance immunogenicity; and possible manipulations in this respect are numerous and varied. Since uptake and appropriate presentation of the antigen is critical to induction of an effective immune response, several groups have modified encoded antigens to target them to for more efficient uptake by profession APCs[60]. Antigens fused to CD40 ligand[61] or the extracellular domain of the Fms-like tyrosine kinase-3 (flt-3) ligand[62], or cytotoxic T-lymphocyte antigen 4 (CTLA4)[63] are examples in which the receptor for each ligand is found on surface of DCs, targeting the antigen to these cells for

an enhanced immune response. Within the cell, the encoded antigen can be modified to promote degradation via the endosomal / lysosomal pathway [64] [65] as a means to enhance antigen presentation in MHC class II and increase CD4+ T cell responses. In a similar approach targeting a different pathway, the proteolytic processing of the encoded antigen can be promoted by fusing it with sequences directing its ubiquitination[66]. Incorporation of heterologous immunogenic sequences, such as a tetanus toxin CTL epitope into a tumor antigen resulted in rapid CTL induction against the tumor antigen with protection against tumor challenge [67]. For human papilloma virus (HPV)-based cancers such as cervical cancer, codon optimization of the antigen has proven an effective means of increasing protein expression and enhancing immune response [68].

One common approach to enhance immunogenicity of nucleic acid vaccines is the co-delivery of DNA encoding cytokines, based on the rationale that a more potent immune response will be elicited if the antigen is presented in a favorable cytokine milieu. To this end, cytokines promoting a Th1 type response, including GM-CSF, IFN-γ, IL-2 and IL-12 have been extensively evaluated in preclinical models of infectious disease[69] and cancer[70] [71], demonstrating the ability of this approach to favorably influence the nature and magnitude of the immune response. Based on the rationale that more efficient delivery of the antigen to APCs will enhance immune responsiveness, chemokines have been used to draw APCs to the site of antigen synthesis. This has been accomplished either by fusion of the antigen to inflammatory chemokines[72] or by co-delivery of the antigen with chemokines[73]. Additional modifications to the region of polynucleotide vaccine delivery includes co-delivery of anti-apoptotic genes to enhance the survival of DNA transfected DCs[74] and co-administration of the antigen-encoding DNA with a soluble lymphocyte activating gene-3 (LAG3) protein as a means to promote cross-presentation of the antigen[75]. *In vivo* expansion of the DCs to enhance immune responsiveness has been directed by delivery of a plasmid encoding the (Flt-3) ligand [76]. This approach can be used in combination with convention peptide vaccines to enhance cellular immune response[77].

Given that self antigens are weakly immunogenic, and that epitope spreading is know to occur upon induction of an immune response, the concept of cross-species homologous immunization, also called xenogenic or orthologous immunization, has proven to be an effective method of breaking tolerance. ForPNI, this strategy uses a tumor antigen gene derived from a different species than the vaccine recipient to induce a cross-reactive immune response to the host autologous protein. For multiple proteins studied to date, the foreign species ortholog displays enhanced immunogenicity as compared

to the autologous or self antigen. This approach leads to immunity that cross-reacts with, and breaks tolerance to, the self antigen. Orthologous immunization has been used successfully in animal models to induce anti-tumor immune responses against either endogenous tumor antigens [78] [79] [80] or tumor promoting factors[81]. Initial clinical studies in prostate cancer using a protein/DC vaccine demonstrated induction of immune response to the self antigen, suggesting the potential utility of this approach in the clinical setting[82].

The ease of preparation and lack of vector-directed immune response associated with DNA vaccines have led to its incorporation into a variety of heterologous prime and boost strategies which have proven more efficacious than DNA immunization alone in a variety of preclinical models and with a variety of different strategies. These have included its use in combination with other genetic vectors[83] [84] or with proteins, for example, adsorbed to PLG microparticles[85]. Although such approaches will be somewhat more complicated to bring to the clinic, the increased potency of combination vaccines may override this consideration.

6. PRECLINICAL STUDIES

The use of appropriate preclinical models is a critical matter for all areas of cancer therapeutics development, and polynucleotide vaccines are no exception. Numerous preclinical models exist, with the majority of work performed in mouse models of cancer. The models have increased in stringency as the field has progressed, reflecting maturation of the technology to more closely approximate the clinical situation. Initially, polynucleotide vaccine strategies for cancer were targeted to artificial tumor antigens such as ovalbumin or beta-galactosidase[15]. Demonstration of tumor protective effects in these model systems led to the development of tumor models in which human tumor antigens, such as carcinoembryonic antigen[86] and MUC-1[87], were transfected into syngeneic mouse tumor cell lines. These studies were useful for proof of principle to demonstrate that CTLs generated to these tumor antigens could protect against a lethal challenge of tumor cells. However, the cross species differences in amino acid composition between the human and mouse rendered these vaccines more immunogenic than what naturally occurs in the clinical setting. More recently, the development of transgenic mouse models expressing human tumor antigens provides more stringent conditions that better recapitulate the clinical scenario under which to optimize polynucleotide vaccines. These models allow investigation into the particular requirements for mounting an effective immune response in the face of existing tolerance to tumor antigens[61] [75]. Another potentially fruitful area of investigation is in the

treatment of companion pets, as recently reported in a study of dogs with spontaneously arising malignant melanoma[88]. The genetic diversity of this population better reflects the scenario that will be encountered with humans. With respect to establishing the safety and feasibility of PNI, experience in large animals and nonhuman primates[89] [90] has been useful to demonstrate the general safety of the approach prior to human clinical trials.

Preclinical models have also proven invaluable in understanding the molecular mechanisms involved in generating an effective antitumor immune response subsequent to PNI. The development of a number mouse models with particular aspects of the immune system have been selectively disrupted (i.e, genetic knockouts) has allowed more clear delineation of the factors critical for the induction of an effective immune response[91]. Investigation of the mechanism of tumor rejection mediated by a therapeutic DNA vaccine in a transgenic mouse model of breast cancer demonstrated the coordinated role of CD4+ and CD8+ cells, antibodies, Fc receptors, perforin, interferon gamma, CD1d-restricted NKT and macrophages, with an important role for activated neutrophils, which may directly lyse cancer cells and affect tumor vasculature[92] [93].

7. Clinical Experience with Polynucleotide Vaccines

While induction of both T and B cell responses to foreign antigens has been convincingly demonstrated in humans with respect to foreign antigens relevant to infectious disease[94] [95] [96] [97] [98] tumor antigens are comparatively weak with respect to antigenicity. Induction of an effective antitumor immune response to such antigens represents a considerable challenge and, to date the clinical experience with polynucleotide vaccines has met with mixed results. The clinical studies have supported the general safety and low toxicity of the vectors, but the potency of the immune response has been disappointing and antitumor efficacy has proven elusive.

Several human clinical trials have been completed recently. Direct intramuscular delivery of DNA encoding a cloned tumor antigen (CEA) has been reported for advanced stage colon cancer[99]. Patients were immunized with a plasmid expressing both CEA and, as a control, hepatitis B surface antigen. Although protective levels of antibodies recognizing the hepatitis protein were detected in some patients, there was little evidence of immune response directed against CEA. Rosenberg and colleagues reported similar findings using a plasmid DNA encoding the melanoma antigen gp100 in a

phase I clinical trial for patients with metastatic melanoma[100]. In this trial of 22 patients immunized either intramuscularly or intradermally, no evidence of gp100-specific immune responses were detected, although one patient exhibited a partial response. The authors concluded that no significant clinical or immunological response was generated. This contrasts with previous clinical trials involving the gp100 antigen delivered as a transgene in a fowlpox-based vaccine or as peptides, and emphasizes the need for strategies to enhance immune response to plasmid DNA vaccines.

Levy and co-worker evaluated the immunogenicity of a plasmid DNA vaccine for patients with B-cell lymphoma[101]. Previous clinical studies using proteins representing tumor-specific immunoglobulin idiotype for active immunization have demonstrated clinical benefit for immunized patients[102] [103]; however preparation of patient-specific protein vaccines is laborious and not feasible for widespread application. DNA vaccination offers the advantage of comparatively rapid and inexpensive preparation. Immunization of patients with a DNA vaccine encoding a chimeric molecule consisting of the patient-specific idiotype fused to the IgG2a and k mouse immunoglobulin constant region chains. Cohorts of patients were immunized with DNA encoding the chimeric vaccine intramuscularly and intradermally using the Biojector needle free delivery device, with or without the addition of plasmid DNA encoding GM-CSF. In all groups of patients, most patients generated an immune response to the murine immunoglobulin carrier protein, demonstrating that the encoded protein was produced and was capable of eliciting an immune response. Induction of an immune response to the Id portion of the encoded gene was infrequent, but was detected in some patients.

It should be noted that these clinical trials were performed in the setting of advanced disease, where induction of an immune response may not be optimal. Nevertheless, collectively the experience with naked DNA transfer in humans for cancer immunotherapy suggests that first generation plasmid DNA vaccines will not be sufficient to elicit a clinically effective immune response against nonmutated self antigens. Translation of the most promising strategies outlined in Table 1 into the clinic may address the limitations of current methods.

Two Phase I trials of DNA vaccines directed against human papilloma virus (HPV)-related malignancies have been reported. Treatment of HPV-related malignancies may offer the advantage of expression of a foreign, HPV antigen in the malignant cells. Plasmid DNA encoding HLA-A2 epitopes from HPV16 E7 protein was encapsulated in a biodegradable polymer microparticles, PLG,

and delivered i.m [104]. This therapeutic trial for individuals with anal dysplasia led to increased T cell responses as detected by ELISPOT in 10 of 12 patients, and partial histological responses in some subjects in the higher dose groups. Use of the same reagent delivered subcutaneously or intramuscularly to women with cervical intraepithelial neoplasia led resulted in detectable immune response to HPV E7 in most patients (73%), and complete histological response in 33% of women[105]. No vaccine related serious adverse events were reported. These studies suggest that DNA vaccines directed at HPV antigens may have a role in management of HPV-related malignancies.

8. Conclusions / Future Directions

The pace of tumor antigen identification has accelerated rapidly in the past few years[106] and will likely increase with new techniques such as expression profiling[107] [108], SEREX[109], and proteomic analysis[110] leading to the identification of new potential targets for active immunotherapy. The use of DNA vaccines in preclinical models may provide a relatively rapid means of evaluating the potential utility of these candidate antigens in mediating tumor rejection. In addition to traditional tumor associated antigens, polynucleotide vaccines may also find a role in vaccines strategies directed against tumor vasculature [111]. Studies of polynucleotide vaccines in the area of infectious disease will continue to be valuable in developing novel strategies that can be incorporated into cancer vaccines. Recent clinical studies targeting infectious disease suggest that prime and boost is also potentiates the immune response in the humans [112]. Although definitive clinical evidence of the efficacy of polynucleotide vaccines in cancer therapy remains to be demonstrated, there is reason to be optimistic about their potential in the management of a wide variety of malignancies. As a relatively nontoxic therapy, PNI may ultimately find its clinical application as an adjuvant in setting of minimal residual disease, where it may be useful in preventing disease recurrence. Eventually, use of polynucleotide vaccines may extend to the cancer prevention arena. The notable advantages of polynucleotide immunization and its proven safety thus far in clinical studies provides a sound basis for their continued development and eventual incorporation into the management of malignant disease.

Table 1. Strategies to Enhance the Efficacy of Polynucleotide Vaccines for Cancer Therapy

Aspect of Vaccine	Intervention	References
Nucleic acid delivery	Liposomes	56
	PLG microparticles	57
	Electroporation	58, 59
Modification of the antigen to target APCs	Fuse antigen with CD40L, Flt-3L, CTLA4	61-63
Modification of the antigen to increase immunogencity	Alter antigen processing	64-66
	Incorporate immunogenic eptiopes	67
	Use antigen from a different species	78-81
	Codon optimization	68
Modification of the microenviroment	Add cytokines	70, 71
	Add chemokines	72, 73
	Decrease apoptosis in APCs	74

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Advances in Brief

CD40 Is Expressed on Ovarian Cancer Cells and Can Be Utilized for Targeting Adenoviruses[1]

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Abstract

Purpose: **CD40, a member of the tumor necrosis factor receptor superfamily, is widely expressed on various cell types in addition to hematopoietic cells. Recent studies show that CD40 expression is related to several carcinomas, although its role in cancer pathobiology is unknown. In this study, we demonstrate the expression of CD40 on several ovarian carcinoma cell lines and the ability of CD40 to mediate targeted adenoviral infection *in vitro*.**

Experimental Design: **CD40 expression on ovarian cancer cell lines and clinical patient samples was examined by reverse transcription-PCR and flow cytometry. To study the utilization of CD40 for gene delivery, we precomplexed a luciferase coding adenovirus (Ad), Ad5luc1, with a CD40-targeting molecule (CAR/G28).**

Results: **According to our studies, all of the examined ovarian cancer cell lines are expressing CD40. In addition, mRNA for CD40 was detected in every primary tumor sample, suggesting that CD40 is also expressed *in vivo*. Compared with nontargeted Ad, gene transfer was increased up to 40-fold in CD40+ cells when CD40-targeted Ad was used. Supporting the relation of targeted system to CD40, increasing the amount of targeting fusion protein results in dose response. Furthermore, blockade of CD40 receptors on cell surface decreases the infectability of CD40+ cells with CD40-targeted virus, indicating the specificity of the targeting system for CD40.**

Conclusions: **These results suggest that CD40 is present in ovarian cancer cells and can be used for targeted gene delivery in a CAR-independent manner, circumventing the problem of the low expression levels of CAR in various cancer cells.**

Introduction

Ovarian cancer causes more deaths than any other cancer of the female reproductive system. In 2001, more than 23,000 new cases were diagnosed, and nearly 14,000 deaths were reported (1). On this basis, it is clear that novel therapeutic strategies are desperately needed. In this regard, cancer gene therapy represents a promising intervention, which embodies the capacity for correction of disorders at a molecular level. All published ovarian cancer gene therapy interventions have exploited adenoviral vectors for *in situ* transduction of tumor cells (2). Specifically, for ovarian cancer, i.p. administration of recombinant Ad[3] has been used for genetic modification of target tumor cells. Although these trials have demonstrated the relative safety of Ad-based delivery of genes for ovarian cancer therapy, they have also demonstrated a low rate of tumor cell transduction. This problem has been attributed to tumor cell deficiency of the primary Ad receptor, CAR (3, 4). Lack of CAR has also been reported for other tumor types and could be a ubiquitous phenomenon (2). As an approach to address this issue, tropism modifications of Ad have been made to allow CAR-independent gene delivery. Such maneuvers have allowed enhancement of gene delivery to otherwise Ad-refractory tumor cells (5). In addition, such modifications may improve the target:nontarget transduction ratio, an important determinant of the therapeutic index. Reported Ad targeting approaches have involved rerouting Ad to cell surface receptors highly expressed in ovarian cancers, including integrins (6), the Ad serotype 3 receptor (7), EpCAM (8), and epidermal growth factor receptor (9). These retargeting approaches embody the concept that tropism modifications of Ad may allow an improved outcome via Ad-based ovarian cancer gene therapy approaches. Such vector engineering efforts will be fostered by the definition of relevant ovarian cancer markers as potential selective targets. In this regard, one potential novel target is CD40, a type I transmembrane protein that belongs to the tumor necrosis factor receptor superfamily (10). It was first identified and characterized on B lymphocytes, and its central role in regulating T-cell-dependent B-cell activation is widely known. However, in recent years, it has been

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[3] The abbreviations used are: Ad, adenovirus; CAR, coxsackie adenovirus receptor; pfu, plaque-forming unit(s); RT-PCR, reverse transcription-PCR; CD40L, CD40 ligand.

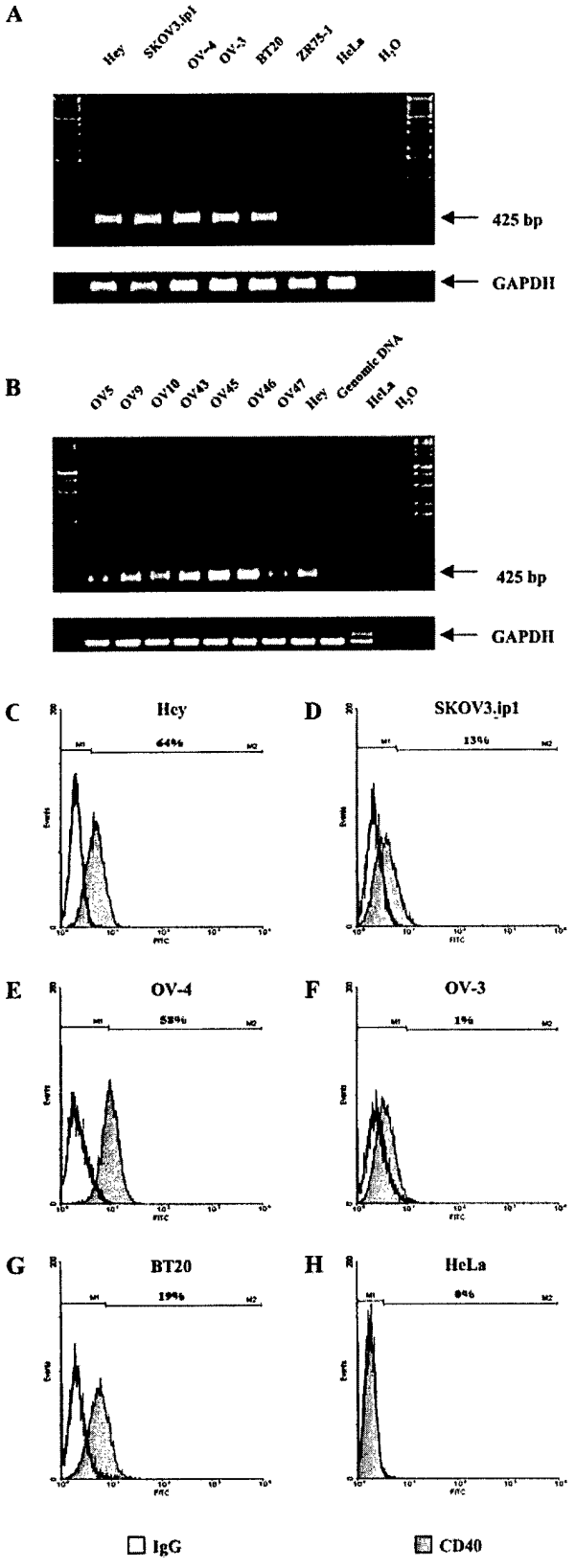

Fig. 1 CD40 expression on ovarian cancer cells. *A*, detection of CD40 mRNA by RT-PCR from Hey, SKOV3.ip1, OV-4, and OV-3 ovarian carcinoma cell lines. BT-20 (CD40+), HeLa (CD40−), and ZR-75-1 (CD40−) were used as controls. *B*, detection of CD40 mRNA by RT-PCR from primary ovarian cancer patient samples. Hey (CD40+), HeLa (CD40−), and genomic DNA were included as controls. CD40-specific primers were used to generate a 425-bp product from isolated total RNA. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers were used to ensure the quality of RNA. *C–H*, CD40 expression levels on ovarian carcinoma cell lines determined by flow cytometry. The *white curve* represents staining with FITC-labeled isotype control antibody, whereas the *gray curve* represents staining with FITC-labeled monoclonal antihuman CD40 antibody. HeLa cells were used as a negative control, and BT-20 cells were used as a positive control. Percentages above the markers indicate proportions of shifted cells.

shown that CD40 is also widely expressed in monocytes, dendritic cells, endothelial cells, epithelial cells, and several types of carcinoma (*e.g.*, breast, lung, colon, and bladder carcinoma), suggesting a role beyond the lymphoid system (10–12). In contrast, the mediator of CD40 activation, CD40L (CD154), is exclusively expressed on activated CD4+ T cells (13). Cellular responses to CD40-CD40L interaction differ based on cell type and range from cell proliferation and differentiation to growth inhibition and apoptotic signaling (12). Even though the presence of CD40 has been suggested on some epithelial malignancies, its exact role in carcinomas still remains unclear (11, 14). Despite this uncertainty, several recent studies show therapeutic implications related to CD40-CD40L interaction. It has been demonstrated that CD40 ligation on carcinoma cells results in growth inhibition and sensitizes cells to apoptosis induced by a variety of agents *e.g.*, chemotherapeutic drugs (14, 15). These contrasting effects of CD40 ligation on normal *versus* malignant cells suggest that CD40 may be an important therapeutic target for antitumoral effect. In the present study, we demonstrate the expression of CD40 in ovarian carcinoma tumor cells. Furthermore, we describe the utilization of CD40 as a candidate pathway for targeted gene transfer via tropism-modified Ad for ovarian cancer gene therapy applications.

Materials and Methods

Cell Culture and Ovarian Tumor Samples. Hey, SKOV3.ip1, and OV-4 ovarian adenocarcinoma cell lines were kind gifts from Dr. Judy Wolf, Dr. Janet Price (both from M. D. Andersson Cancer Center, Houston, TX), and Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA), respectively. The other cell lines, OV-3 (ovarian adenocarcinoma), HeLa (cervical cancer), and BT-20 and ZR-75-1 (breast cancers), were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured as recommended. Ovarian tumor samples were collected from patients undergoing surgical evaluation at the University of Alabama at Birmingham for suspected epithelial ovarian carcinoma or primary peritoneal carcinoma. Permission to obtain specimens was reviewed and approved by the University of Alabama at Birmingham Institutional Review Board for Human Experimentation, and informed consent was given before surgery. Specimens were collected sterilely at the time of surgery and immediately frozen at −70°C.

RT-PCR. Total RNA was isolated from cultured cells using the RNeasy Kit (Qiagen, Valencia, CA) and treated with DNase before RT-PCR. Amplification (35 cycles,

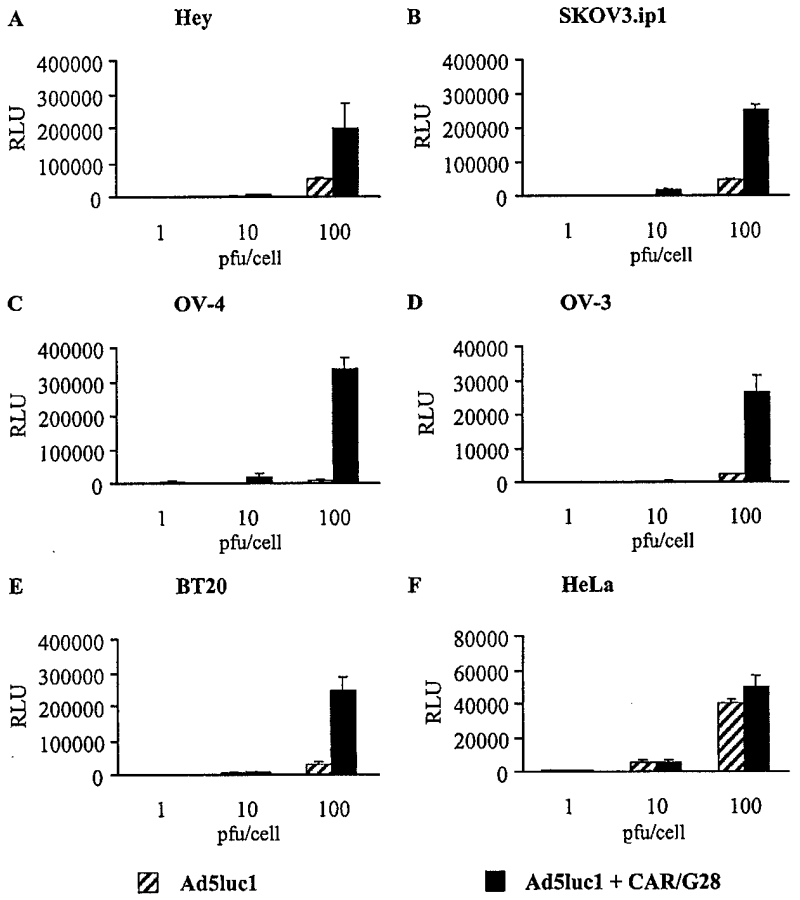

Fig. 2 Infectivity of CD40-expressing cell lines is enhanced when CD40 retargeted Ad is used. Cells were infected with Ad5luc1 (*striped column*) or with CAR/G28 retargeted Ad5luc1 (*black column*). Luciferase readings were measured 24 h after infection. BT-20 (CD40+) and HeLa (CD40−) were used as controls. The *error bars* indicate ±1 SD.

annealing at 56°C) was carried out with the OneStep RT-PCR Kit (Qiagen) using CD40-specific primers (upstream, 5′-AGA-AGG-CTG-GCA-CTG-TAC-GA-3′; downstream, 5′-CAG-TGT-TGG-AGC-CAG-GAA-GA-3′). For amplification of glyceraldehyde-3-phosphate dehydrogenase (upstream, 5′-TCC-CAT-CAC-CAT-CTT-CCA-3′; downstream, 5′-CAT-CAC-GCC-ACA-GTT-TCC-3′), 30 amplification cycles were performed (annealing at 52°C). Total RNA from ovarian tumor samples was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer's instruction. RT-PCR was performed as described above.

Flow Cytometry. After trypsinization, 1×10^5 cells were resuspended in fluorescence-activated cell sorting buffer (PBS containing 2% fetal bovine serum) and stained either with FITC-conjugated mouse antihuman CD40 monoclonal antibody or FITC-conjugated IgG1 isotype control antibody (BD Biosciences, San Diego, CA). Cells were incubated at 4°C for 20 min and washed with fluorescence-activated cell sorting buffer before flow cytometric analysis (FACScan; Becton Dickinson, San Jose, CA).

Gene Transfer Assays. CAR/G28 fusion protein containing an anti-CD40 single chain Fv and the CAR ectodomain was made as described previously (16). Briefly, anti-CD40 single chain Fv cDNA generated from the G28-5 hybridoma cell line was linked to cDNA of the CAR ectodomain, resulting in CAR/G28 fusion protein. CAR/G28 was produced using recombinant baculoviruses, purified, and characterized. Replication-incompetent luciferase-expressing Ad Ad5luc1 (3.9×10^{12} viral particles/ml, 5.5×10^{10} pfu/ml; Ref. 7) was incubated with CAR/G28 retargeting protein before infections for 45°C at room temperature. Cells were infected with either nontargeted or targeted virus for 1 h at 37°C, followed by washing. Cells (25,000 cells/well) were seeded in 96-well plates 1 day before infections and then infected with nontargeted or CAR/G28-targeted virus at a ratio of 100 ng/100 pfu. Luciferase assay was performed with the Luciferase Assay System (Promega, Madison, WI) 24 h after infections, following the manufacturer's instructions. In the dose-response assay, virus was preincubated with different amounts of CAR/G28 fusion protein (0, 3, 10, 30, 100, or 150 ng/100 pfu). Infections were carried out on 24-well plates using 50,000 cells/well. Luciferase assay was performed as described above.

Blocking Assay. Before infections, OV-4 and BT-20 cells were incubated with either growth media (containing 2% fetal bovine serum), supernatant from G28-5 hybridoma cells containing monoclonal antihuman CD40 antibody (blocking) (16), or supernatant from 1D11.16.8 hybridoma cells containing monoclonal anti-transforming growth factor β2 antibody (irrelevant antibody). Infections were carried out on 24-well plates as described above.

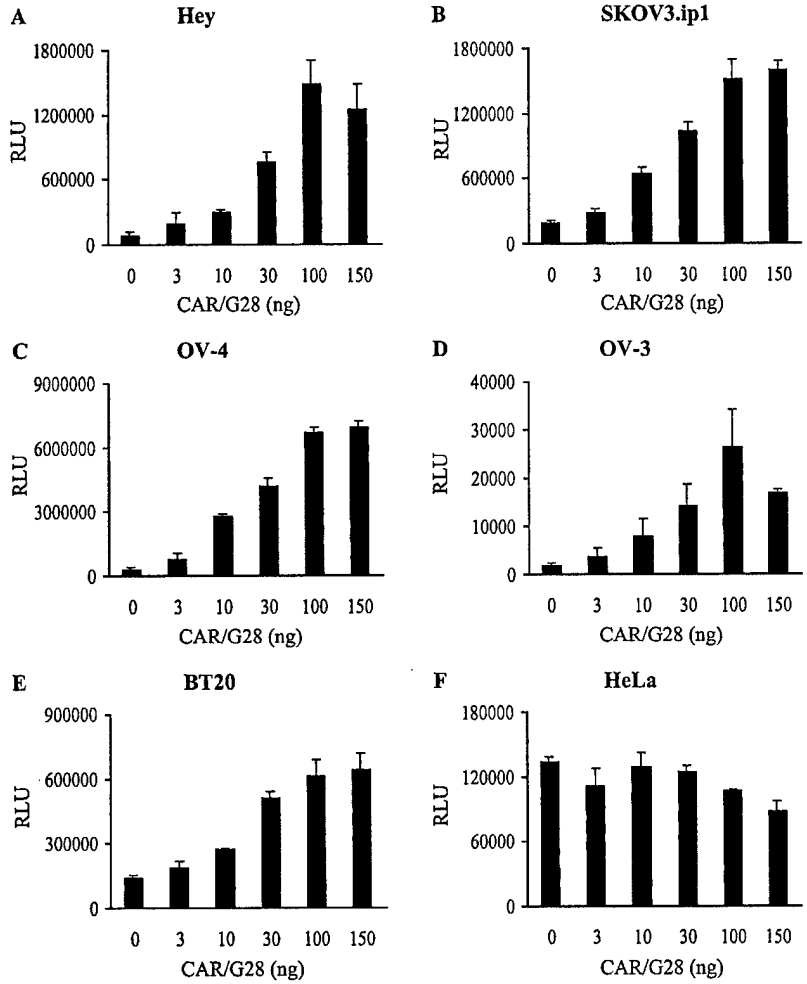

Fig. 3 Increasing the amount of retargeting protein (CAR/G28) results in dose response in CD40+ cells. Ad5luc1 was preincubated with 0–150 ng/100 pfu CAR/G28, and luciferase assay was performed after 24 h. BT-20 (CD40+) and HeLa (CD40−) cell lines were used as controls. The *error bars* indicate ±1 SD.

Results and Discussion

Although the biological role of CD40 in carcinoma cells is mostly unknown, several studies have focused on CD40 expression in various cancer types (11, 15, 17, 18). In contrast to B cells, CD40-CD40L interaction in carcinoma cells has been shown to prevent cell growth and sensitize cells to apoptosis (11, 14, 15). Additionally, some studies have suggested CD40 as a potential marker to distinguish benign tumors from malignant tumors (17). On this basis, we wanted to study CD40 expression in the context of ovarian cancer. RT-PCR was performed with total RNA isolated from various ovarian carcinoma cell lines (Fig. 1*A*). All of the examined cell lines (Hey, SKOV3.ip1, OV-4, and OV-3) and the positive control cell line (BT-20) generated a 425-bp RT-PCR product, indicating that all of the above-mentioned cells contain CD40 mRNA. ZR-75-1 and HeLa have been reported as negative for CD40 expression (11, 14), and they did not produce any amplification product. To study the presence of CD40 on the cell surface, cells were analyzed by flow cytometry using a FITC-labeled anti-CD40 antibody with an appropriate isotype control (Fig. 1, *C–H*). Based on previous studies (11, 14) and results from the RT-PCR, BT-20 (Fig. 1*G*) was chosen as a positive control, and HeLa (Fig. 1*H*) was chosen as a negative one. HeLa cells, as expected, did not show any expression when stained with CD40 antibody, whereas in the case of BT-20, almost 20% of cells were gated as CD40 positive, demonstrating moderate CD40 expression. The four examined ovarian carcinoma cell lines (Fig. 1, *C–F*) displayed variable but positive CD40 expression levels. Both Hey (Fig. 1*C*) and OV-4 (Fig. 1*E*) cells expressed high levels of CD40, with approximately 60% of cells being gated as CD40 positive. CD40 was also detected on SKOV3.ip1 (Fig. 1*D*) and OV-3 (Fig. 1*F*) cells, but to a lesser extent, suggesting moderate and low expression of CD40, respectively.

It has been suggested that cells negative for CD40 expression *in vivo* could revert to a CD40-positive phenotype when cultured *in vitro* (12). To examine the CD40 status in unpassaged human primary ovarian cancer cells, RT-PCR was done on primary tumor samples obtained from seven patients. RT-PCR resulted in the expected 425-bp band with all samples, suggesting CD40 expression (Fig. 1*B*). Genomic DNA did not yield amplification product, suggesting the absence of pseudogenes or other sources of false positives. These results provide the first evidence of CD40 expression in clinical ovarian tumor specimens.

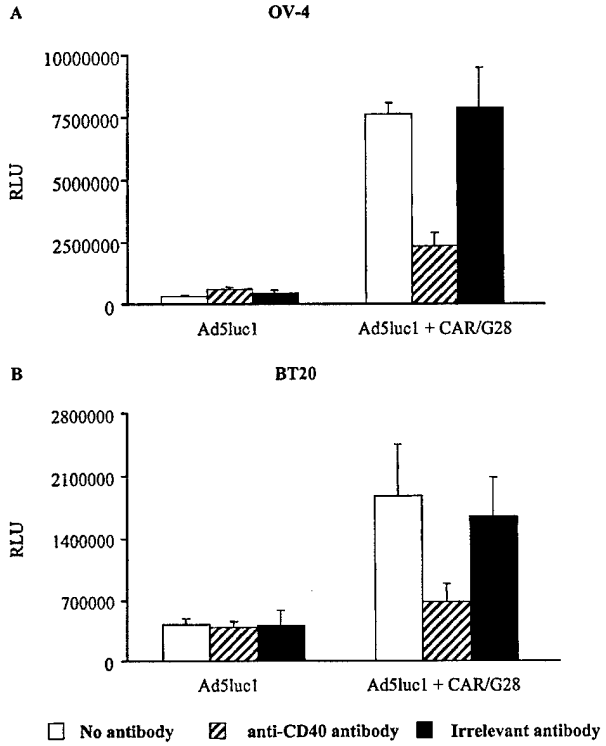

Fig. 4 Blockade of CD40 receptors decreases the infectivity of CD40 retargeted Ad in CD40+ cells. Cells were treated with growth media containing no antibodies (*white columns*), monoclonal anti-CD40 antibody (*striped columns*), or an irrelevant monoclonal antibody (*black columns*) before infections with Ad5Luc1 or Ad5Luc1 + CAR/G28. Luciferase activity was determined at 24 h after transduction, and *error bars* indicate ±1 SD.

There is increasing recent evidence suggesting lack of the primary Ad receptor, CAR, on ovarian (3, 4) and other types of cancer cells (2). Unfortunately, CAR expression is the major factor determining infection efficacy with Ad serotype 5 (2, 7). Therefore, several approaches have been tried to circumvent dependence on CAR (6–9). Having established CD40 expression on ovarian cancer cells, we investigated the potential of this phenomenon for targeting Ad. First, cells were infected with three doses of nontargeted (Ad5luc1) or CD40-targeted (Ad5luc1 + CAR/G28) Ads (Fig. 2). With the CD40-negative HeLa cells (Fig. 1), CAR/G28 conferred no advantage in gene expression. (Fig. 2*F*). In contrast, all CD40-positive cell lines (Fig. 2, *A*–*E*) demonstrated notable enhancement in gene transfer efficacy when CD40-targeted virus was used. In comparison with Ad5Luc1 alone, gene transfer was increased 4-, 6-, 42-, 13-, and 8-fold for Hey, SKOV3.ip1, OV-4, OV-3, and BT-20 cells, respectively, when 100 pfu/cell Ad5Luc1/CAR/G28 was used (Fig. 2).

To confirm that increased gene expression was due to the retargeting moiety, a second set of experiments was performed, in which the viral dose was kept constant, whereas the amount of fusion protein was increased (Fig. 3). In HeLa cells (negative control), the increase of retargeting protein did not cause a dose response (Fig. 3*F*). With the CD40-positive cell lines, increasing CAR/G28 resulted in a dose-dependent increase in luciferase expression (Fig. 3, *A*–*E*). With some cell lines, the higher amounts of fusion protein (100 and 150 ng) may have saturated the Ad fibers available for binding the native receptor CAR, resulting in plateauing of the transgene expression. However, with cells expressing high levels of CD40, such as OV-4, the full retargeting potential was not reached. These results suggested that utilization of CD40 for increased transduction allowed enhanced transgene expression in cells low in CAR, such as these ovarian cancer cells (7)

Conceivably, the augmentation of transgene expression seen in the experiments described in Figs. 2 and 3 was due to CAR/G28-mediated binding, followed by internalization with the usual penton base RGD-cellular integrin-mediated mechanism. Because ovarian cancer cells typically express low levels of CAR (4, 7), including the cells used here, a higher frequency of CD40 receptors (Fig. 1) thus allowed increased binding, entry, and consequent marker gene expression. To confirm the dependence of transgene expression on CAR/G28-CD40 interaction, we preincubated cells with an anti-CD40 antibody. This blocked binding of the CAR/G28-targeted Ad and reverted luciferase expression close to levels achieved without targeting (Fig. 4). An irrelevant antibody, anti-transforming growth factor β2, had no effect on targeted transgene expression. Furthermore, these experiments excluded the remote possibility that mere binding of CD40 would increase transgene expression to the degree seen here (Fig. 4, AdLuc1, *striped bars*).

The results of this study suggest that ovarian cancer cell lines and patient samples express CD40. Therefore, ovarian cancer is a potential target for CD40L therapy, which has been shown to cause apoptosis and suppress tumor growth *in vitro* and in animal models (11, 13–15). Moreover, we propose and demonstrate the feasibility of a strategy whereby CD40 is used for targeted gene delivery with adenoviral vectors. Although more experiments are required to confirm the feasibility of CD40 targeting *in vivo*, utilization of tumor associated receptors, such as CD40, could help to increase transduction of target tissues, resulting in augmentation of the clinical efficacy of adenoviral cancer gene therapy approaches (5). Furthermore, this could reduce the total dose required for antitumor efficacy. Importantly, targeting complexes have also demonstrated the potential for reducing transduction of nontarget tissues including the liver, which is the main organ responsible for Ad clearance and therefore a potential source of side effects (19, 20). Yet another application could be the use of CD40-targeted Ads in immunotherapeutic approaches, *e.g.*, modification of dendritic cells more efficient with reduced viral doses (16).

In conclusion, we have demonstrated high expression levels of CD40 on ovarian cancer cell lines and clinical primary tumor specimens. Then, we used a fusion protein for targeting Ad vectors to CD40 and, in comparison with untargeted Ad, we observed increased transgene expression, which has been shown to correlate with increased Ad transduction efficiency (16). Targeting to tumor-associated receptors could help improve the efficacy while reducing the side effects of cancer gene therapy approaches.

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